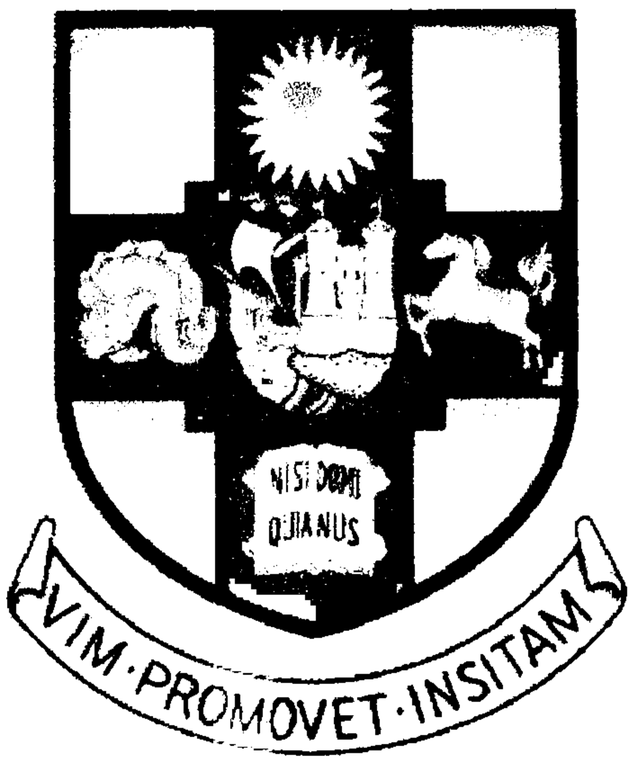
Author:
Talbot, Helen Marie

Title:
Steryl chlorin esters : origin, significance and potential as indicators of phytoplankton community structure.

STERYL CHLORIN ESTERS: ORIGIN, SIGNIFICANCE AND POTENTIAL AS INDICATORS OF PHYTOPLANKTON COMMUNITY STRUCTURE

HELEN MARIE TALBOT

A thesis submitted to the University of Bristol
in accordance with the requirements of the degree of
Doctor of Philosophy in the Faculty of Science



Environmental and Analytical Section
School of Chemistry
University of Bristol
Cantock's Close
Bristol BS8 1TS

July 1999

ABSTRACT

Steryl chlorin esters (SCEs) are significant chlorophyll transformation products and preliminary evidence has indicated they are herbivory products. Further investigation of the sources of SCEs and the relationship between SCE sterol distributions and those of their precursors (algal and animal) have been carried out. Production of SCEs *a* (pyrophaeophorbide *a* nucleus) was demonstrated in feeding experiments where the copepod *Calanus helgolandicus* grazed on cultures representing the four major algal divisions: *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Chlamydomonas reginae* (Chlorophyta); *Pleurochrysis carterae* (Haptophyta); *Thalassiosira weissflogii* Bacillariophyta); *Prorocentrum micans* and *Alexandrium tamarensis* (Dinophyta). SCEs *b* (pyrophaeophorbide *b* nucleus) occurred in the *T. suecica* experiment. No SCE production was observed with two other haptophytes (*Isochrysis galbana*, *Cocolithus pelagicus*). Where SCEs occurred, in all but one experiment (*P. carterae*) each of the available algal sterols was present in the SCE fraction. Comparison of the algal sterol distributions and SCE sterols revealed that, where cholesterol was not a significant component of the substrate (*P. carterae*, *T. weiss*), significant alteration of the substrate distribution occurred prior to esterification. With the dinoflagellate, discrimination against the uptake of 4-methyl sterols into the SCE fraction was observed. Pellet ageing studies showed a significant degradation of both SCEs and other chlorins but that the SCE sterol distribution remained constant (unlike the free sterols). Overall, the results suggest that sedimentary SCE sterols are not quantitative indicators of phytoplankton populations, but that they should be of value in assessing changes in such populations.

Other transformation products not previously detected in faecal pellets were found. The bicyclic chlorin, 13^2-hydroxychlorophyllone *a*, previously only associated with diatoms and thought to be the precursor of certain bicyclic sedimentary porphyrins, occurred in aged pellets (*T. weiss*) and in the algal control and pellets (*A. tamarensis*). It is thought to have been concentrated during grazing and pellet ageing, thereby explaining its significant abundance in sediments. SCE production was not observed during grazing of a heterotrophic dinoflagellate *Oxyrrhis marina* feeding on a green alga or *I. galbana* and of a mixed community of small mesozooplankton on a cyanobacterium. However, *O. marina* afforded a number of known and novel transformation products including 13^2-hydroxychlorophyllone *a*, 13^2-hydroxy-chlorophyllone *b*, 13^2-oxopyrophaeophorbide *a* and purpurin-18 which are thought to have been produced after cellular disruption due to grazing.

ACKNOWLEDGEMENTS

Firstly I would like to thank Prof. James Maxwell for his supervision, support and encouragement during this work. I am also grateful to Dr Roger Harris, Bob Head and Cilla Course (PML) for introducing a chemist to the fascinating world of marine biology and all of their help with the feeding experiments. Penny, Isabelle, Xabier, Betina and Dave are thanked for spending many an hour in the cold room "chasing" copepods. I would like to than Dr Paul Harradine, Dr. Christine Reiss, Dr. Valerie Cariou-Le Gall and Dr. Anke Putschew for their help and guidance at the start of this work. Jim Carter and Andy Gledhill are thanked for their help and patience with the mass spectrometers. Many thanks to Sue Trott for all of her help and encouragement, not to mention always being there with a smile and time to chat, keeping me going when times were tough.

Matt S. and Hazel, thanks for many a *serious* discussion setting the world to rights over the odd pint (or 4, or 5…) of Guinness! Special thanks go to Anthony, Matt F., Ian, Paul, Gordon and all fellow true believers: remember "Understanding is a three edged sword". Many thanks go to all of the other members of the labs and other drinking partners I have met during my time in Bristol: Andy S., Kath, Martin, Lizzy, Nick, Steve, Matt H., Mark, Andy T., Cheggs, Gareth, Helen, Simon W., Suzie, Steph, Rob, Luke, Chris, Andy M., Ian M., Matt L., Alex, Simon M., Sophie, Vicky, Zoe, Sarah and so many more……

Loraine, thankyou for being there when I needed you and for providing such a great place to live. Richard and Chris, thanks for your patience and faith and Alan, if it wasn't for you I wouldn't have made it this far. Ann-Marie, Fiona, Rebecca, Helen and Annette, thankyou for your continued friendship and support even when my letter writing is less than consistent.

Adam, where would I be without you keeping me up to date with news from north of the border and reminding me what it's really all about!!

Finally I would like to thank my mum and sister for all their love and support (and Joanne and Dave, thanks for the best excuse for a holiday ever) and Dad - I miss you.

DECLARATION

I hereby certify that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this or any other University.

The views expressed in this thesis are solely those of the author and not of the University of Bristol.

Helen M. Talbot

CONTENTS

ABSTRACT		**i**
ACKNOWLEDGEMENTS		**ii**
DECLARATION		**iii**
PREFACE		**xx**
NOMENCLATURE AND NUMBERING		**xxii**
GLOSSARY		**xxiii**
Chapter 1.	**INTRODUCTION**	**1**
1.1.	Background	**2**
1.2.	Water Column Transformations of Chlorophyll	**3**
1.2.1.	General	**3**
1.2.2.	Herbivory	**5**
1.2.3.	Senescence	**8**
1.2.4.	Oxidative Transformations	**10**
1.2.5.	Other Transformations	**11**
1.2.6.	Steryl Chlorin Esters	**12**
1.2.6.1.	Occurrence	**12**
1.2.6.2.	Mode of Formation	**14**
1.2.6.3.	Potential Significance	**17**
1.2.6.4.	Related Products	**19**
1.2.6.5.	Summary	**21**
1.3.	Present Study	**21**
Chapter 2.	**FEEDING EXPERIMENTS WITH GREEN ALGAE**	**23**
2.1.	Introduction	**24**
2.1.1.	Previous Work	**24**
2.1.2.	Diversity of Production of SCEs and Relationship to Substrate	**24**
2.2.	Present Study	**25**
2.2.1.	Background	**25**

2.2.2.	Occurrence of Chlorophyll *b*	**25**
2.2.3.	Feeding Experiments	**26**
2.3.	Stage 1: Results and Discussion	**29**
2.3.1.	Algal Cultures	**29**
2.3.2.	Faecal Pellets	**31**
2.3.3.	Summary	**34**
2.4.	Stage 2: Results and Discussion	**35**
2.4.1.	Algal Culture	**35**
2.4.2.	Algal Control	**43**
2.4.3.	Faecal Pellets	**50**
2.4.4.	Free Sterols	**50**
2.4.4.1.	Pre-Starved Copepods	**50**
2.4.4.2.	Algal Culture	**51**
2.4.4.3.	Fed Copepods	**52**
2.4.4.4.	Comparison of Free and SCE Sterol Relative Abundance	**53**
2.5.	Discussion	**56**
2.5.1.	Pigments	**56**
2.5.2.	Sterols	**57**
2.6.	Summary	**59**
Chapter 3	**FEEDING EXPERIMENTS WITH HAPTOPHYTES**	**61**
3.1.	Introduction	**61**
3.1.1.	The Haptophyceae	**61**
3.1.2.	Photosynthetic Pigments	**62**
3.2.	Feeding Experiments	**62**
3.3.	Results	**63**
3.3.1.	Algal Culture	**63**
3.3.2.	Algal Control	**68**
3.3.3.	Faecal Pellets	**68**
3.3.4.	Free Sterols	**73**
3.3.4.1.	Pre-Starved Copepods	**73**
3.3.4.2.	Algal Culture	**73**

3.3.4.3.	Fed Animals	**76**
3.3.4.4.	Comparison of Free and SCE Sterol Relative Abundance	**76**
3.4.	Discussion	**78**
3.4.1.	Pigments	**78**
3.4.2.	Sterols	**80**
3.5.	Summary	**82**
Chapter 4	**FEEDING EXPERIMENTS WITH A DIATOM**	**83**
4.1.	Introduction	**84**
4.1.1.	Background	**84**
4.1.2.	Present Study	**84**
4.1.3.	The Bacillariophyta (Diatoms)	**85**
4.2.	Results: Preliminary Experiment	**86**
4.2.1.	Pigments	**86**
4.2.2.	Sterols	**88**
4.2.3.	Summary	**89**
4.3.	Results: Large Scale Experiment	**90**
4.3.1.	Algal Culture	**90**
4.3.2.	Algal Control	**91**
4.3.3.	Fresh Faecal Pellets (0 d)	**92**
4.3.4.	Faecal Pellets (10 d)	**92**
4.3.5.	Faecal Pellets (30 d)	**96**
4.3.6.	SCEs	**97**
4.3.7.	Free Sterols	**99**
4.3.7.1.	Starved Animals	**99**
4.3.7.2.	Culture	**99**
4.3.7.3.	Fed Animals	**100**
4.3.7.4.	Comparison of SCE and Free Sterol Distributions	**100**
4.3.8.	Ageing Studies	**102**
4.4.	Discussion	**103**
4.5.	Summary	**110**

Chapter 5	**FEEDING EXPERIMENTS WITH DINOFLAGELLATES**	**111**
5.1.	Introduction	**112**
5.1.1.	Background	**112**
5.1.2.	The Dinophyta	**113**
5.1.3.	Present Study	**113**
5.2	Results: Small Scale *P. micans*	**115**
5.2.1.	Algal Culture	**115**
5.2.2.	Faecal Pellets	**115**
5.2.3.	SCEs	**116**
5.2.4.	Sterols	**117**
5.2.4.1.	*P. micans* Culture	**117**
5.2.4.2.	Comparison of SCE and Culture Sterols	**118**
5.2.5.	Summary	**119**
5.3.	Results: *A. tamarensis* Experiment	**120**
5.3.1.	Algal Culture	**120**
5.3.2.	Algal Control	**121**
5.3.3.	Faecal Pellets	**122**
5.3.4.	Sterols	**124**
5.3.5.	Summary	**125**
5.4.	Results: Large Scale *P. micans*	**126**
5.4.1.	Algal Culture	**126**
5.4.2.	Algal Control	**127**
5.4.3.	Faecal Pellets (0 d)	**128**
5.4.4.	Faecal Pellets (8 d)	**128**
5.4.5.	Faecal Pellets (29 d)	**128**
5.4.6.	Sterilised Faecal Pellets (29 d)	**129**
5.4.7.	SCEs	**130**
5.4.8.	Sterols	**132**
5.4.8.1.	Starved Animals	**132**
5.4.8.2.	Algal Culture	**133**
5.4.8.3.	Faecal Pellets	**135**

5.4.8.4.	Comparison of SCE and Culture Free Sterols	**136**
5.4.9.	Ageing Studies	**137**
5.4.9.1.	SCEs	**137**
5.4.9.2.	Sterols	**138**
5.5.	Discussion	**140**
5.5.1.	Small Scale Experiments	**140**
5.5.2.	Large Scale Experiment	**140**
5.6.	Summary	**145**
Chapter 6	**FEEDING EXPERIMENTS WITH A HETEROTROPHIC DINOFLAGELLATE AND A SMALL MESOZOOPLANKTON POPULATION**	**146**
6.1.	Introduction: Microzooplankton Experiments	**147**
6.1.1.	Background	**147**
6.1.2.	Present Study	**149**
6.2.	Introduction: Mesozooplankton Experiment	**151**
6.2.1.	Background	**151**
6.2.2.	Present Study	**152**
6.3.	Results: *O. marina* and Green Alga	**153**
6.3.1.	Green Alga	**153**
6.3.2.	*O. marina* (and some green alga cells)	**154**
6.3.3.	Feeding Experiment	**157**
6.4.	Results: *O. marina* and *I. galbana*	**157**
6.4.1.	Culture	**157**
6.4.2.	Feeding Experiment (fed 2 d prior to harvesting)	**159**
6.4.3.	Feeding Experiment (fed 8 d prior to harvesting)	**160**
6.5.	Discussion: Microzooplankton Experiments	**160**
6.6.	Results: Mesozooplankton Experiment	**164**
6.6.1.	Fresh Culture (0 h)	**164**
6.6.2.	Fresh Culture (24 h)	**164**
6.6.3.	Feeding Experiment	**165**
6.7.	Discussion: Mesozooplankton Experiment	**166**

6.8.	Summary	**166**
Chapter 7	**GENERAL DISCUSSION AND FUTURE WORK**	**168**
7.1.	General Discussion	**169**
7.1.1.	Aims	**169**
7.1.2.	Formation of Steryl Chlorin Esters	**169**
7.1.3.	Comparison of SCE and Substrate Sterol Distributions	**171**
7.1.3.1.	Algal Cholesterol Content	**172**
7.1.3.2.	4-Methyl Sterols	**176**
7.1.4.	Pellet Ageing	**176**
7.1.5.	Pellet Sterilisation	**178**
7.1.6.	Alternative Chlorin Nuclei	**178**
7.1.7.	SCE Sterol Distributions *vs*. Free Sterol Distributions	**180**
7.1.8.	Other Chlorin Products	**181**
7.1.8.1.	Allomers	**181**
7.1.8.2.	Purpurins	**184**
7.1.8.3.	13^2-Hydroxychlorophyllone *a*	**185**
7.1.8.4.	13^2-Oxopyrophaeophorbide *a*	**187**
7.1.8.5.	Formation of Oxygenated Products in Copepod and Protozoan Experiments	**188**
7.1.8.6.	General Significance and Potential as Grazing Indicators	**188**
7.2.	Future Work	**190**
7.2.1.	General	**190**
7.2.2.	Further Copepod Studies	**190**
7.2.3.	Other Experiments	**193**
Chapter 8	**EXPERIMENTAL**	**195**
8.1.	General	**196**
8.1.1.	Glassware	**196**
8.1.2.	Solvents	**196**
8.2.	Feeding Experiments	**196**
8.2.1.	Copepod Experiments	**196**

8.2.1.1.	Pellet Ageing (large scale *T. weiss* experiment 1b)	**199**
8.2.1.2.	Pellet Ageing (normal scale *P. micans* experiment 2a)	**199**
8.2.1.3.	Pellet Ageing (large scale *P. micans* experiment 2b)	**199**
8.2.2.	*Oxyrrhis marina* Feeding Experiments	**200**
8.2.3	Microzooplankton (500-200μm) and Cyanobacterium Feeding Experiment	**200**
8.3.	Extraction of Samples	**201**
8.4.	Pigment Analysis	**201**
8.4.1.	High Performance Liquid Chromatography	**201**
8.4.2.	High Performance Liquid Chromatography - Mass Spectrometry	**202**
8.5.	Sterol Analysis	**203**
8.5.1.	Free Sterol Isolation	**203**
8.5.2.	Derivatisation	**203**
8.5.3.	Gas Chromatography	**203**
8.5.4.	Gas Chromatography - Mass Spectrometry	**203**
STRUCTURES		**204**
REFERENCES		**211**

LIST OF FIGURES

Figure 1-1.	Diagenetic scheme for the conversion of chlorophyll *a* to DPEP based on identification of individual components (after Keely *et al.*, 1990)	**4**
Figure 1-2.	24-ethyl-4α-methyl-5α(H)-cholestan-3β-yl pyrophaeophorbide *a* (Prowse and Maxwell, 1991)	**13**
Figure 2-1.	Outline of samples and analysis protocols for Stage 2 large scale *C. helgolandicus* and *T. suecica* feeding experiment	**28**
Figure 2-2.	LC-MS base peak traces from copepod feeding on *Dunaliella tertiolecta*	**29**
Figure 2-3.	LC-MS base peak traces from copepod feeding on *Chlamydomonas reginae*	**29**
Figure 2-4.	LC-MS base peak traces from copepod feeding on *Tetraselmis suecica*	**30**
Figure 2-5.	APCI mass spectra of (a) chl *a* and (b) chl *b*	**30**
Figure 2-6.	Mass spectra of (a) pyrophaeophytin *a* and (b) pyrophaeophorbide *a*	**32**
Figure 2-7.	Mass spectra of (a) pyrophaeophytin *b* (co-eluting with phaeophytin *a*) and (b) pyrophaeophorbide *b*	**32**
Figure 2-8.	Selected mass chromatograms from *T. suecica* faecal pellet SCE region	**33**
Figure 2-9.	Mass spectra of selected peaks in SCE region of *T. suecica* faecal pellets: (a) SCE *a*, MH^+= 915 - C_{28} sterol with 2 double bonds; (b) Possible SCE *b*, MH^+= 929; (c) Possible SCE *b*, MH^+= 917; (d) Unknown high molecular weight peak.	**34**
Figure 2-10.	HPLC chromatograms (400 + 430 nm) from large scale *T. suecica* (prasinophyte) feeding experiment	**35**
Figure 2-11.	Mass and electronic spectra of peak 4 (chl *a*)	**36**
Figure 2-12.	Mass and electronic spectra of peak 4' (chl *a'*)	**36**
Figure 2-13	Mass and electronic spectra of peak 12 (13^2-OH chl *a* and 15^1-OH chl *a* lactone)	**37**

Figure 2-14	Mass and electronic spectra of peak 3 (chl *b*)	**38**
Figure 2-15	Mass and electronic spectra of peak 3' (chl *a'*)	**28**
Figure 2-16	Mass and electronic spectra of peak 10 (15^1-OH chl *b* lactone)	**38**
Figure 2-17	Mass and electronic spectra of peak 11 (13^2-OH chl *b*)	**39**
Figure 2-18	Mass and electronic spectrum of peak 8 (phaeophytin *a*)	**39**
Figure 2-19	Mass and electronic spectrum of peak 8' (phaeophytin *a'*)	**40**
Figure 2-20	Mass and electronic spectrum of peak 6 (13^2-hydroxy-phaeophytin *a*)	**40**
Figure 2-21	Mass and electronic spectrum of peak 6' (13^2-hydroxy-phaeophytin *a'*)	**40**
Figure 2-22	Mass and electronic spectrum of peak 5 (phaeophytin *b*)	**41**
Figure 2-23	Mass and electronic spectrum of peak 9 (pyrophaeophytin *a*)	**43**
Figure 2-24	Mass and electronic spectrum of peak 2 (pyrophaeophorbide *a*)	**43**
Figure 2-25	Mass and electronic spectrum of peak 7 (pyrophaeophytin *b*)	**44**
Figure 2-26	Mass and electronic spectrum of peak 1 (pyrophaeophorbide *b*)	**44**
Figure 2-27	Mass and electronic spectra of peak 12 (faecal pellet extract; 13^2-OH chl *a* and 15^1-OH chl *a* lactone)	**45**
Figure 2-28	Mass and electronic spectra of peak 13 (15^1-methoxy- and hydroxy phaeophytin *a* lactone)	**46**
Figure 2-29	Mass and electronic spectrum of peak 15 (purpurin-18-phytyl ester)	**46**
Figure 2-30	HPLC chromatograms (400 nm and 430nm) and mass chromatograms of faecal pellet SCE region	**47**
Figure 2-31	Mass and electronic spectra of SCE peak a (single pyrophaeophorbide *b* ester MH^+=915)	**48**
Figure 2-32	Mass and electronic spectra of SCE peak b (two co-eluting pyrophaeophorbide *b* ester MH^+=915 and 929)	**48**
Figure 2-33	Mass and electronic spectra of SCE peak c (single pyrophaeophorbide *b* ester MH^+=917)	**49**
Figure 2-34	Mass and electronic spectra of SCE peak d (1 pyrophaeophorbide *b* ester MH^+=931and 1 pyrophaeophorbide *a* ester MH^+=901)	**49**

Figure 2-35	Mass and electronic spectra of SCE peak e (two co-eluting pyrophaeophorbide *a* ester MH^+=901 and 915)	**49**
Figure 2-36	Mass and electronic spectr of SCE peak c (single pyrophaeophorbide *a* ester MH^+=903)	**49**
Figure 2-37	Mass and electronic spectra of SCE peak c (single pyrophaeophorbide *a* ester MH^+=903)	**49**
Figure 2-38	Partial GC-MS RIC traces of free sterols (asTMSi ethers) in pre-starved copepods, algal culture and fed copepods. (Unlabelled peaks are non-sterol.)	**50**
Figure 2-39	Mass spectra of pre-starved *C. helgolandicus* sterols (TMSi ethers)	**51**
Figure 2-40	Mass spectra of pre-starved *T. suecica* sterols (TMSi ethers)	**52**
Figure 2-41	Relative % of algal sterols in SCEs *a*, SCEs *b* and culture	**53**
Figure 2-42	Relative % of animal sterols in SCEs *a*, SCEs *b*, starved and fed copepods	**55**
Figure 2-43	Mechanism of sterol C_{24} dealkylation operating in insects and probably crustaceans (Goad, 1978, 1981; Svoboda and Feldlaufer, 1991)	**58**
Figure 3-1	HPLC chromatograms (410 nm) from copepod feeding on *P. carterae*	**64**
Figure 3-2	Mass and electronic spectra of peak 15	**65**
Figure 3-3	Mass and electronic spectra of peak 16	**66**
Figure 3-4	Mass and electronic spectra of peak 17	**67**
Figure 3-5	Mass and electronic spectra of peak 18	**67**
Figure 3-6	Mass and electronic spectra of peak 19	**68**
Figure 3-7	Mass and electronic spectra of peak 20 (phaeophytin *a* lactone-like	**69**
Figure 3-8	Mass and electronic spectra of peak 21	**69**
Figure 3-9	Mass and electronic spectra of peak 22	**70**
Figure 3-10	Faecal pellet SCE region mass chromatograms and HPLC chromatogram (410 nm), shaded peaks are SCE MH^+	**71**
Figure 3-11	Mass and electronic spectra of SCE peak a	**71**

Figure 3-12	Mass and electronic spectra of SCE peak b	**72**
Figure 3-13	Mass and electronic spectra of SCE peak c	**72**
Figure 3-14	Mass and electronic spectra of SCE peak d	**72**
Figure 3-15	Mass and electronic spectra of SCE peak e	**72**
Figure 3-16	Mass and electronic spectra of SCE peak f	**72**
Figure 3-17	Partial RIC trace of *P. carterae* free sterols (as TMSi ethers)	**73**
Figure 3-18	Mass spectra of *P. carterae* sterols (as TMSi ethers)	**75**
Figure 3-19	Relative % of animal sterols in SCEs, starved and fed copepods	**76**
Figure 3-20	Relative % of algal sterols in SCEs and culture	**78**
Figure 4-1	Outline of samples and analysis protocols for large scale *T. weiss* feeding experiment	**85**
Figure 4-2	Mass chromatograms of SCE MH^+ from aged pellets	**87**
Figure 4-3	Relative abundance of SCEs in preliminary *T. weiss* experiment (For sterol assignments see large scale experiment section 4.3.7.)	**88**
Figure 4-4	LC-MS base peak chromatograms from large scale *T. weiss* experiment	**90**
Figure 4-5	Mass spectrum of peak 24	**91**
Figure 4-6	Base peak chromatograms of bulk (4 beaker) pellet samples highlighting minor chlorin components present at all stages of ageing	**92**
Figure 4-7	Mass and electronic spectra and proposed structure of peak 25	**93**
Figure 4-8	Mass and electronic spectra of peak 26	**94**
Figure 4-9	Mass and electronic spectra and structure of peak 27 (13^2-oxopyrophaeophytin *a*?)	**95**
Figure 4-10	Mass and electronic spectra and structure of peak 28 (13^2-oxopyrophaeophorbide *a* 24-methylcholesta-5,24(28)-dien-3β-ol?)	**95**
Figure 4-11	Mass chromatograms showing relative retention times of the *R* and *S* C-13^2 epimers of 13^2-hydroxychlorophyllone *a* (peaks 29 and 29') and pyrophaeophorbide a (peak 2)	**96**
Figure 4-12	Mass spectra of (a) peak 29 (13^2-OH chlorophyllone *a*) and (b) peak 29' (13^2-*epi*-OH chlorophyllone *a*)	**96**

Figure 4-13	Absorbance (400 nm) and mass chromatograms of faecal pellet SCE region (10 d sample)	**97**
Figure 4-14	Mass and electronic spectra of SCE peak a (MH^+=901)	**98**
Figure 4-15	Mass and electronic spectra of SCE peak b (MH^+=901 and 915)	**98**
Figure 4-16	Mass and electronic spectra of SCE peak c (MH^+=929)	**98**
Figure 4-17	Mass and electronic spectra of SCE peak d (MH^+=903)	**98**
Figure 4-18	Mass and electronic spectra of SCE peak e (MH^+=917 and 929)	**99**
Figure 4-19	Mass and electronic spectra of SCE peak f (MH^+=931)	**99**
Figure 4-20	Free and SCE sterol relative abundances	**100**
Figure 4-21	Faecal pellet SCE relative abundance with ageing (based on LC-MS mass chromatogram peak areas)	**102**
Figure 4-22	(a) mass of SCE per pellet, (b) SCE % of total chlorins	**102**
Figure 4-23	Dealkylation pathway for conversion of C-24 substituted sterols to cholest-5-en-3β-ol	**104**
Figure 4-24	Aetioporphyrin III	**106**
Figure 4-25	Conversion of chl *a* to 13^2-OH chlorophyllone *a* (adapted from Watanabe *et al.*, 1993)	**108**
Figure 4-26	Examples of sedimentary chlorins and porphyrins with seven membered exocyclic ring	**108**
Figure 4-27	Suggested pathway for production of novel components (peaks 27 and 28)	**109**
Figure 5-1	Outline of samples and analysis protocols for large scale *P. micans* and *C. helgolandicus* feeding experiment	**114**
Figure 5-2	HPLC chromatograms (400 nm) from copepod feeding on *Prorocentrum micans* (small scale experiment)	**115**
Figure 5-3	Absorbance (400 nm) and mass chromatograms from faecal pellet SCE region (small scale *P. micans* experiment)	**116**
Figure 5-4	Partial RIC trace of free sterols (as TMSi) ethers) in algal culture (unlabelled peaks are non-sterol)	**117**
Figure 5-5	Relative % of SCE and algal sterols	**118**
Figure 5-6	LC-MS base peak traces from copepod feeding on *A. tamarensis*	**120**

Figure 5-7	Mass spectra of (a) peak 29 (13^2-hydroxychlorophyllone *a*)and (b) peak 29' (13^2-*epi*-hydroxychlorophyllone *a*)	**121**
Figure 5-8	Mass spectra of (a) peak 30 (13^2,17^3-cyclophaeophorbide *a* enol) and (b) peak 27 (13^2-oxopyrophaeophytin *a*)	**122**
Figure 5-9	Mass spectra of (a) peak 14 (purpurin-18-phytyl ester) and (b) peak 18	**123**
Figure 5-10	Mass chromatograms of faecal pellet SCE region	**123**
Figure 5-11	Mass spectra of (a) SCE peak a (MH^+=903) and (b) SCE peak b (MH^+=945)	**124**
Figure 5-12	Comparison of SCE and culture free sterol relative abundance	**124**
Figure 5-13	HPLC chromatograms (400 nm) from large scale *P. micans* feeding experiment	**126**
Figure 5-14	Mass spectrum of peak 33	**127**
Figure 5-15	Mass spectrum of peak 34	**128**
Figure 5-16	HPLC chromatogram (400 nm) of sterilised ($HgCl_2$) faecal pellets	**129**
Figure 5-17	Faecal pellet SCE region mass chromatograms (shaded peaks are SCE MH^+)	**130**
Figure 5-18	Mass spectrum of SCE peak a (MH^+=915)	**131**
Figure 5-19	Mass spectrum of SCE peak b (MH^+=903)	**131**
Figure 5-20	Mass spectrum of SCE peak c (MH^+=929)	**131**
Figure 5-21	Mass spectrum of SCE peak d (MH^+=931)	**132**
Figure 5-22	Mass spectrum of SCE peak e (MH^+=945)	**132**
Figure 5-23	Mass spectrum of SCE peak f (MH^+=947)	**132**
Figure 5-24	Partial RIC traces of pre-starved animals, culture and faecal pellet free sterols (as TMSi ethers). (Unlabelled peaks are non-sterol)	**133**
Figure 5-25	*P. micans* culture free sterol distribution (*see fig. 5-24)	**134**
Figure 5-26	Mass spectra of *P. micans* 4-desmethyl sterols (see fig. 5-24)	**134**
Figure 5-27	Mass spectra of *P. micans* 4-methyl sterols (see fig. 5-24)	**135**
Figure 5-28	Comparison of SCE and culture free sterol distributions	**136**
Figure 5-29	Faecal pellet SCE relative abundance with ageing (based on peak areas in HPLC 400 nm chromatogram	**137**

Figure 5-30	(a) Mass SCEs per pellet; (b) SCEs % of total chlorins	**138**
Figure 5-31	Relative distribution of free sterols in faecal pellets with ageing	**138**
Figure 5-32	(a) Mass of sterol per pellet, (b) Ratio of sterols to SCEs in pellets	**139**
Figure 6-1	Outline of *O. marina* and green alga experiment. (Key: i. total mixture filtered directly; ii. aliquot separated into 2 fractions.)	**150**
Figure 6-2	Outline of *O. marina* and *I. galbana* experiment. (Key: i. fed 8 d prior to harvesting; ii. fed again 2 d prior to harvesting.)	**151**
Figure 6-3	HPLC chromatograms (400+430 nm) from *O. marina* and green alga experiment (* indicates carotenoid)	**153**
Figure 6-4	Mass and electronic spectra of peak 29 (13^2-hydroxy-chlorophyllone *a*)	**154**
Figure 6-5	Mass and electronic spectra of peak 29+23 (13^2-hydroxy-chlorophyllone *a* and phaeophorbide *a*)	**154**
Figure 6-6	Mass and electronic spectra of peak 35 (13^2-hydroxy-chlorophyllone *b*)	**155**
Figure 6-7	Mass and electronic spectra of peak 36 (13^2-oxopyro-phaeophorbide *a*)	**156**
Figure 6-8	Electronic spectra of (a) peak 37 (purpurin-18) and (b) peak 2 (pyrophaeophorbide *a*) and mass spectrum of (c) peaks 37 and 2	**156**
Figure 6-9	Electronic spectra of (a) peak 38 and (b) peak 39	**157**
Figure 6-10	HPLC chromatograms (400 nm) from *O. marina* and *I. galbana* experiment (* indicates carotenoid)	**158**
Figure 6-11	Electronic spectrum of peak 40	**159**
Figure 6-12	Electronic spectra of (a) peak 41 and (b) peak 42 and (c) mass spectrum of combined peaks	**160**
Figure 6-13	Transformation pathway for chl *a* operating in *O. marina* feeding experiments	**163**
Figure 6-14	HPLC chromatograms (400 nm) from mesozooplankton (200-500 μm) feeding on fresh *Synechococcus* sp. culture	**165**

Figure 7-1	Mechanism of chl *a* allomerisation proposed by Hynninen (1991); adapted from Woolley *et al.* (1998); for brevity, only ring E is shown	**182**
Figure 7-2	Conversion of chl a to an aetioporphyrin	**184**
Figure 8-1	Map of location of site L4 (English Channel, UK)	**198**

LIST OF TABLES

Table 1-1.	Algal species used in copepod feeding experiments	22
Table 2-1.	Chlorin data from large scale *T. suecica* experiment.	42
Table 2-2.	MH^+ ions of pyrophaeophorbide *a* and *b* esters and identity of esterifying sterol	47
Table 2-3.	SCE data from large scale *T. suecica* experiment	55
Table 3-1.	Pigment data for *P. carterae* experiment	65
Table 3-2.	SCE MH^+ and corresponding sterol	70
Table 3-3.	*P. carterae s*terol assignments and structures	74
Table 3-4.	SCE data for *P. carterae* experiment	77
Table 4-1.	SCE MH^+ and corresponding sterol	87
Table 4-2.	SCE sterols and structures (* see figs. 4-4 and 4-13)	101
Table 5-1	SCE MH^+ and corresponding esterified sterol (* see fig. 5-3)	117
Table 5-2	SCE MH^+ and assignment of corresponding sterol	118
Table 5-3	SCE MH^+ and corresponding esterified sterol	131
Table 5-4	Sterol assignments and structures (*see fig. 5-24)	135
Table 5-5	SCE MH^+ and corresponding esterified sterol	136
Table 6-1	Pigment data for noteworthy components in *O. marina* and green alga experiment	155
Table 7-1	Algal species used in experiments which demonstrated the production of SCEs during grazing by the copepod *C.* helgolandicus	169
Table 7-2	SCE % total chlorins in faecal pellets	170
Table 7-3	Abundance of 4-methyl sterols in algal substrates and SCEs	176
Table 7-4	Summary of chl transformation products detected in feeding experiment products	183
Table 8-1	Copepod feeding experiments	197
Table 8-2	Typical gradient elution program for HPLC	202
Table 8-3	Gradient elution program for HPLC of pigment samples from *T. suecica* experiment (6b)	202

PREFACE

This thesis is presented in eight chapters. Following the introduction (Chapter 1), Chapters 2-5 describe laboratory herbivory experiments in which a copepod was allowed to graze on algal cultures from the four major algal divisions to determine which species are suitable substrates for steryl chlorin ester (SCE) production and what factors influence the sterol distribution observed in the SCEs. Chapter 2 details four experiments with three species of Chlorophyta for investigation of the production of steryl chlorin esters containing the pyrophaeophorbide *b* moiety. Chapter 3 concerns three herbivory experiments using haptophyte substrates. Chapter 4 describes two experiments with a diatom species and includes analysis of herbivory products over a period of ageing. Chapter 5 details three experiments with 2 species of dinoflagellate intended to investigate possible steric effects during production of 4-methyl sterol SCEs and also describes changes in free and SCE sterol abundance and distribution in faecal pellets during ageing. Chapter 6 describes two experiments investigating the production of chlorophyll transformation products during microzooplankton grazing and one involving grazing of a cyanobacterium by a "small size" mesozooplankton population. Chapter 7 involves a general discussion of the results obtained and proposals for future work. Chapter 8 details the experimental and analytical procedures employed in this work.

Numbers in bold after a compound refer to its structure as given in the appendices.

Publications:

Pearce, G.E.S., Harradine, P.J., Talbot, H.M. and Maxwell, J.R. (1998) Sedimentary sterols and steryl chlorin esters: distribution differences and significance. *Organic Geochemistry* **28**, 3-10.

Kowalewska, G., Winterhalter, B. Talbot, H.M., Maxwell, J.R. and Konat, J. (1999) Chlorins in sediments of the Gotland Deep (Baltic Sea) *Oceanologia* **41**, 81-97.

Talbot, H.M., Head, R.N., Harris, R.P. and Maxwell, J.R. (1999) Distribution and stability of steryl chlorin esters in copepod faecal pellets from diatom grazing. *Organic Geochemistry*. In press.

Talbot, H.M., Head, R.N., Harris, R.P. and Maxwell, J.R. (1999) Steryl esters of pyrophaeophorbide *b*: A sedimentary sink for Chlorophyll *b*. *Organic Geochemistry*. In press.

Talbot, H.M., Head, R.N., Harris, R.P. and Maxwell, J.R. (1999) Discrimination against 4-methyl sterol incorporation during steryl chlorin ester formation. Submitted.

Talbot, H.M., Head, R.N., Harris, R.P. and Maxwell, J.R. (1999) How suitable are steryl chlorin esters as indicators of phytoplankton community structure? In preparation.

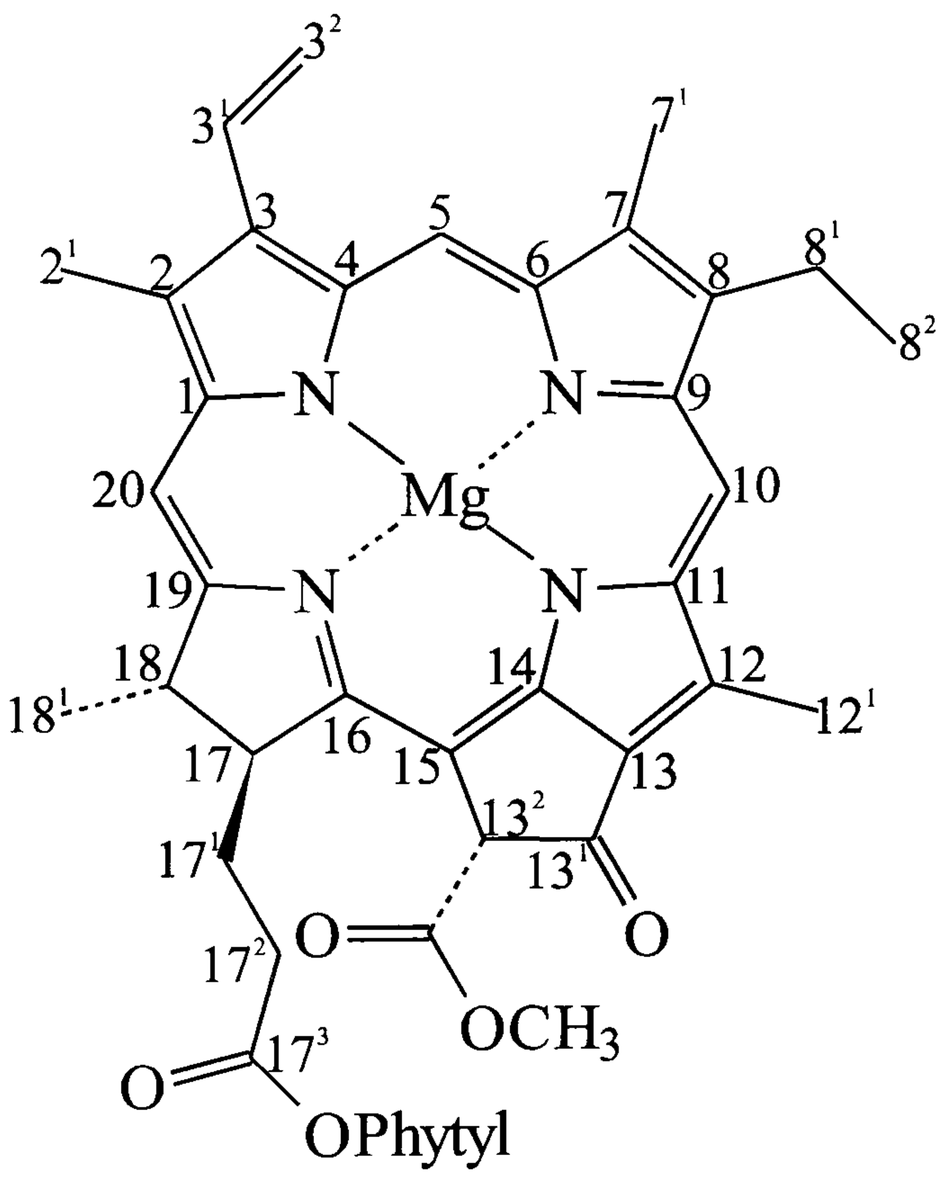
Talbot, H.M., Head, R.N., Harris, R.P. and Maxwell, J.R. (1999) Production of chlorophyll transformation products during grazing by the marine heterotrophic dinoflagellate *Oxyrrhis marina*. In preparation.

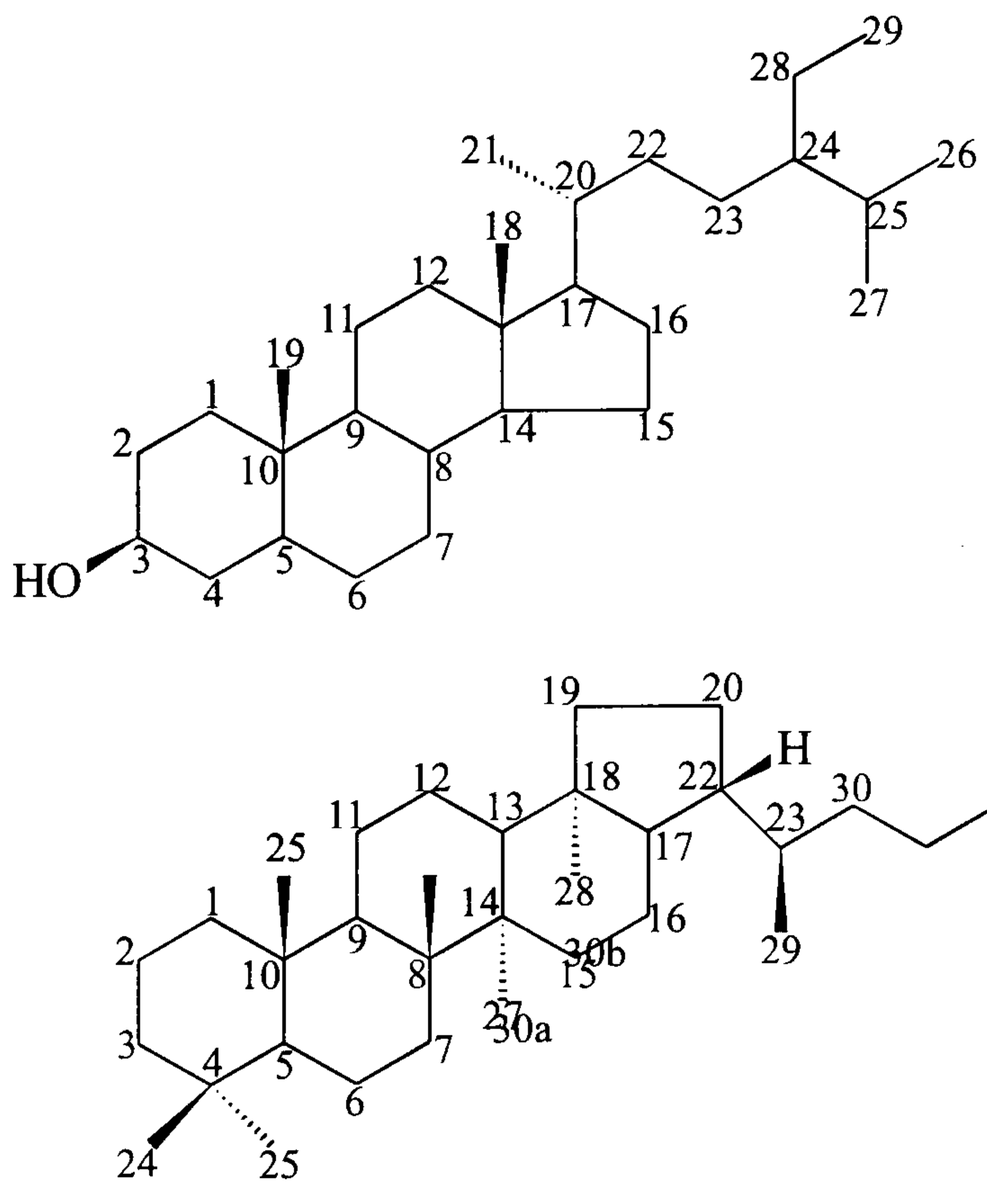
NOMENCLATURE AND NUMBERING

In this thesis the term chlorin is used to refer to a dihydroporphyrin. The following trivial nomenclature is used for the transformation products of chlorophylls:

Phaeophytin:	A free base counterpart of a chlorophyll
Phaeophorbide:	A de-esterified phaeophytin
Pyro:	A component lacking the C-13^2 carbomethoxy group
Meso:	A component in which the C-3 vinyl group is reduced

The IUPAC numbering system for chlorins, sterols and hopanoids is used throughout:





GLOSSARY

APCI	atmospheric pressure chemical ionisation
CAP	cycloalkano porphyrin
CBE	chlorophyll bleaching enzymes
chl	chlorophyll
DCM	dichloromethane
DPEP	desoxophylloerythroaetioporphyrin
GC	gas chromatography
FAB	fast atom bombardment
GF/F	glass fibre filter (fine)
HMW	high molecular weight
HPLC	high performance liquid chromatography
LC	(high performance) liquid chromatography
Me	methyl
MeO	methoxy
MeOH	methanol
MS	mass spectrometry
NMR	nuclear magnetic resonance
PDA	photodiode array (detector)
POM	particulate organic matter
RIC	reconstructed ion current
SCE	steryl chlorin ester
SCE *a*	steryl chlorin ester (containing pyrophaeophorbide *a* moiety)
SCE *b*	steryl chlorin ester (containing pyrophaeophorbide *b* moiety)
spm	suspended particulate matter
TLC	thin layer chromatography
TMSi	trimethylsilyl

Chapter 1

INTRODUCTION

1.1. BACKGROUND

Chlorophylls are the green tetrapyrrolic pigments present in all phototrophic organisms and are essential catalysts in the process of photosynthesis. There are approximately 25 known chlorophylls, the most abundant of which, chlorophyll *a* (chl *a*, **I**), is found in all oxygenic photosynthetic eukaryotes, Cyanobacteria (blue green algae) and some prokaryotic prochlorophytes (Jeffrey and Vesk, 1997). In eukaryotes chl *a* is often accompanied by lower concentrations of chlorophyll *b* (chl *b*, **II**) or the chlorophylls *c* (chl *c*, **III, IV, V**) and other accessory pigments, carotenoids, which help to broaden the spectral range which an organism can utilise for photosynthesis.

Chlorophyll concentration is one of the most frequently measured biochemical parameters in oceanography, having been used as a unique marker for oceanic plant biomass (phytoplankton) for over 40 years (Jeffrey and Mantoura, 1997 and references therein). Chlorophyll measurements can track diurnal, seasonal and longer term changes in productivity in aquatic environments. Analysis of chls and associated carotenoid pigments can provide information about the chemotaxonomic compositions of phytoplankton populations as well as provide information about the flux, transformation and ultimate fate of these pigments and their degradation products (e.g. Repeta and Gagosian, 1987; Barlow *et al.*, 1993).

Interest in the study of chls and carotenoids as biological markers has developed significantly since the introduction of high performance liquid chromatography methods for the rapid and precise determination of pigment signatures (e.g. Mantoura and Llewellyn, 1983). Subsequent improvements (e.g. Wright *et al.*, 1991 and references therein) and the applications of other techniques such as ^{1}H NMR (e.g. Keely *et al.*, 1987; Keely and Maxwell, 1991) and mass spectrometry (e.g. Eckardt *et al.*, 1990; Keely and Maxwell, 1991; Harris *et al.*, 1995a) have increased understanding of both the sources and fates of phytoplankton pigments.

1.2. WATER COLUMN TRANSFORMATIONS OF CHLOROPHYLL

1.2.1. General

Global chl degradation is *ca.* 10^9 tonnes each year and most of this degradation (*ca.* 75%) occurs in oceans, lakes and rivers (Hendry *et al.*, 1987; Porra *et al.*, 1997). It appears that the proportion of chl and its derivatives that survive long enough to be removed from the photic zone with the chromophore intact is very small (Brown *et al.*, 1991) and only *ca.* 0.1% is preserved in the sedimentary record (*e.g.* Callot, 1991).

The two main reaction pathway types involved in the degradation of chlorophyll are known as Type I and Type II reactions (Brown *et al.*, 1991). Type I reactions involve modification or removal of the peripheral substituents of the macrocycle whilst the chromophore remains intact. In aquatic systems transformation products include chlorophyllides (loss of esterifying alcohol), phaeophytins (loss of Mg) and phaeophorbides (loss of Mg and phytol) and their pyro counterparts (loss of the C-13^2 carbomethoxy group); these are formed by herbivorous grazing (see sections 1.2.2 and 1.2.6) and by cell senescence (see section 1.2.3) producing a series of products, some of which are shown in Figure 1-1. This scheme shows the minimum number of intermediates necessary to link chl *a* to desoxophylloerythroaetioporphyrin (DPEP, **VI**), the most abundant porphyrin in ancient sediments (e.g. Keely *et al.*, 1990). This relationship was first suggested by Treibs (1936) based on the structural similarities between chl *a* and DPEP. The ability to link sedimentary tetrapyrroles to their major precursors, the chls, can provide important information with regard to assessment of the conditions at the time of deposition and hence palaeoclimate reconstruction (e.g. Eckardt *et al.*, 1990, 1991a).

Type II reactions involve oxidative cleavage of the macrocycle (photooxidation) followed by further degradation to lower molecular weight colourless compounds (see section 1.2.4.). These reactions in which the chromophore is lost occur in the upper layers (photic zone) of oceans and rivers and are responsible for the largest proportion of chlorophyll degradation (e.g. Brown *et al.*, 1991).

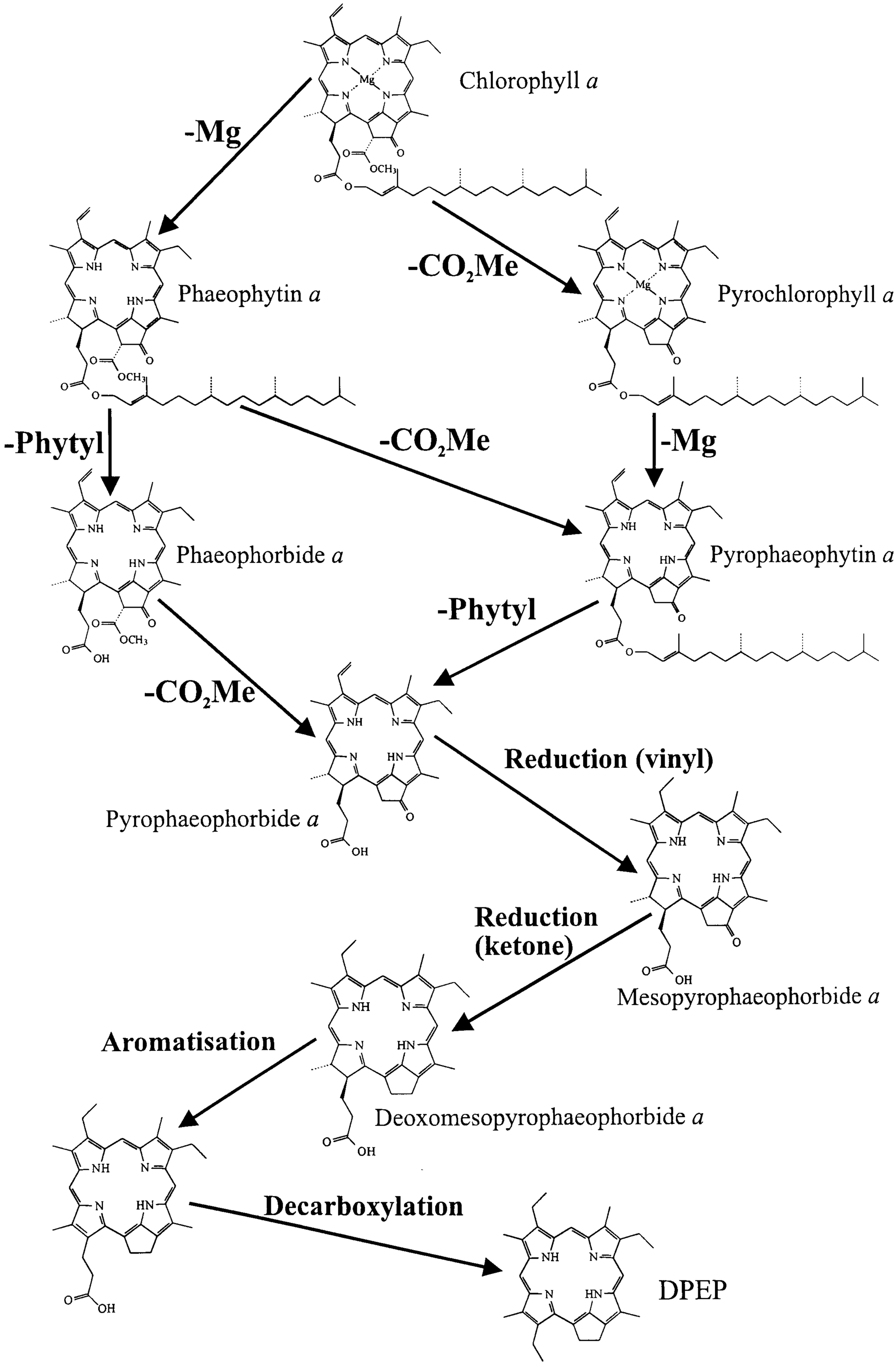


Figure 1-1. *Diagenetic scheme for the conversion of chlorophyll a to DPEP based on identification of individual components (after Keely et al., 1990).*

1.2.2. Herbivory

Zooplankton herbivory is the most important process associated with the production of sedimentary chlorophyll transformation products in which the chlorophyll macrocycle is still intact. Rapidly sinking faecal pellets from zooplankton are known to be an important transport mechanism for labile organic molecules, including chl-derived compounds, from the upper zones of the oceans to the benthos (e.g. Welschmeyer and Lorenzen, 1985) where the largest proportion of this material is then often utilised by benthic organisms with only a minor proportion being incorporated in to the sediment (e.g. Lorenzen *et al.*, 1981; Callot, 1991). However, the proportion of marine carbon flux directly attributable to grazing i.e. faecal pellet flux, can vary significantly depending on a variety of factors, with reported values for pellet contribution to total material flux ranging from 1% (Pilskaln and Honjo, 1987) to >60% (e.g. Dunbar and Berger, 1981). In general, larger zooplankton produce larger and often more compact faecal pellets which have higher sinking rates than those produced by smaller species and consequently are transported out of the photic zone, where the majority of pellet remineralisation occurs, more rapidly than smaller particles (e.g. Butler and Dam, 1994 and references therein). Pellet sinking rate can also vary according to phytoplankton diet taxonomic composition, with those containing a high proportion of phytoplankton theca (e.g. coccoliths, diatom frustules) having higher sinking rates than pellets derived from soft bodied flagellates (e.g. Bienfang, 1980; Harris, 1994). Pellet production rates vary with location in that average production rates in areas influenced by upwelling are often 2-3 times greater than those in more oligotrophic areas (Pilskaln and Honjo, 1987).

The contribution of faecal pellet material to the sedimentary environment may also be retarded for smaller pellets which have been shown to be rapidly colonised by bacteria which degrade the peritrophic membrane (Gauld, 1957) enclosing the pellet, causing dispersal of the pellet material in a relatively short time in warm water (a few hours at 20 °C; Caron *et al.*, 1989); consequently it is thought that much of the faecal material produced by copepods in surface waters does not reach the deep oceans (Hofmann *et al.*, 1981). However, as copepods are amongst the most abundant mesozooplankton species their contribution is still significant. It is also possible for pellets to be scavenged by

larger particles such as faecal strings and faecal aggregates of gelatinous zooplankton to produce rapidly sinking "marine snow" flocs which are thought to contribute significantly to particulate matter flux in the deep ocean (Pilskaln and Honjo, 1987). Small faecal pellets may also contribute to the diet of larger zooplankton such as the filter feeding salps which graze primarily on particulate organic matter (POM). The larger, denser faecal pellets of macrozooplankton are thought to be less susceptible to microbial invasion and decomposition (Caron *et al.*, 1989), their higher sinking rates ensuring rapid removal from the photic zone and limiting loss of pigments by photolysis.

The presence of chlorins or phaeopigments in zooplankton faecal material was first reported by Currie (1962). There has since been intense interest in many aspects of zooplankton grazing, including prey selection, ingestion rate, pellet production rate/size/density, pellet lipid signatures and pigment content. An early report (Shuman and Lorenzen, 1975) suggested that conversion of chl *a* to phaeophorbide *a* (**VII**) was 100% efficient on a molar basis. Later reports found that phaeophytin *a* (**VIII**) was also a significant component of faecal material (e.g. Hallegraeff, 1981), however, chlorophyllide *a* (**IX**) does not seem to be a significant product of herbivory, being more commonly associated with senescence (see below). More recently, it has been shown that conversion is not always complete; some proportion of chl *a* may pass through the gut unchanged (e.g. Downs and Lorenzen, 1985; Head and Harris, 1992). In other cases some chl is degraded to non fluorescent compounds in the gut (e.g. Conover *et al.*, 1986; Dagg and Walser, 1987; Kiørboe and Tiselius, 1987; Lopez *et al.*, 1988; Pasternak and Drits, 1988; Penry and Frost, 1991; Head and Harris, 1996). Head and Harris (1996) showed that significant chl *a* destruction occurs prior to entry into the guts of the copepod (i.e. as soon as cells are macerated by the copepod) and proposed that this destruction could be explained in terms of an enzymatic process involving "chlorophyll bleaching enzymes" (CBEs) which may cause oxidation and cleavage of the macrocyclic ring (*cf.* Brown *et al.*, 1991). The CBE activity was found to increase with increasing ingestion rate suggesting that enzymes derived from the algae themselves were contributing to the chl destruction during grazing (see section 1.2.3.). At low ingestion rates CBEs in the copepod appeared to be able to destroy all or most of

the ingested chl, although previous feeding history of the copepod was also a factor (e.g. Penry and Frost, 1991), whilst at higher ingestion rates a greater proportion of the total destruction was due to the phytoplankton enzymes. At high ingestion rates the degree of chl destruction tended to a minimum of *ca.* 45% (Head and Harris, 1996). Other reports of the extent of chlorophyll destruction vary significantly (10-100%), however, although very high destruction efficiencies would seem to be atypical with 10-30% being average for copepods (Kiørboe and Tiselius, 1987).

Baker and Louda (1986) stated that phaeophorbide and phaeophytin are the major chl derivatives generated during water column passage, and that loss of the carbomethoxy group to form pyrochlorins occurs in suboxic sediments. However, Hawkins *et al.* (1986) found that pyrophaeophorbides and pyrophaeophytins are produced by the mussel *Mytilus edulis* during grazing and a more recent study (Head and Harris, 1992) found that the most concentrated pigment in copepod faecal pellets was pyrophaeophorbide *a* (**X**), constituting 44-71% of the total identifiable pigments, with pyrophaeophytin *a* (**XI**) also being a significant component. Otsuki *et al.* (1993) have also shown pyrochlorins to be significant components of the faecal pellets of bentho-pelagic malacostracan crustaceans, indicating another source of pyrochlorins in sediments.

Although similar products have been shown to be formed by both meso and macro zooplankton, the situation involving microzooplankton (e.g. heterotrophic dinoflagellates, ciliated protozoa) is significantly different. A number of studies have shown that the production of chl derivatives in which the chromophore is intact does not seem to occur under all circumstances (e.g. Klein *et al.*, 1986; Barlow *et al.*, 1988; see also Chapter 6). Strom (1991, 1993), however, has demonstrated that, at least under certain conditions, protozoa can produce significant quantities of chlorins, some of which would seem to differ from those generally associated with macro and mesozooplankton grazing (discussed in more detail in Chapter 6). The conflicting nature of these results does indicate that the use of chl breakdown products as an estimate of grazing activity may not provide an accurate indication of the extent of activity in

natural populations of zooplankton containing microzooplankton, leading to underestimation of total grazing rates (e.g. Barlow *et al.*, 1988).

1.2.3. Senescence

Senescence is a term widely used to describe the onset of cell death brought about by a variety of processes, including genetically programmed ageing, cell lysis (both natural through grazing or bacterial action or artificially by ultrasonication or osmotic shock treatment), lack of nutrients or prolonged exposure to darkness. During senescence, chl defunctionalization occurs and is generally thought to be enzymatic in nature. Under certain conditions the chl transformation products delivered to the sediment are more likely to be produced by senescent processes. These conditions include direct settling of dead phytoplankton cells from a bloom when there is little or no zooplankton grazing, zooplankton performing "sloppy feeding" (Roy *et al.*, 1989; Head and Harris, 1996) or the release of senescent cells from ice melts (Louda *et al.*, 1998 and references therein).

The enzyme chlorophyllase is known to catalyse the hydrolysis of the esterifying side chain to form chlorophyllides *in vivo* (e.g. and Jeffrey, 1964). This enzyme has been shown to be present in a large number of algal species and is particularly common in diatoms, leading Jeffrey and Hallegraeff (1987) to propose that chlorophyllide *a* (**IX**) could be a marker of senescent diatoms, although it has been observed in the products of grazing experiments (e.g. Downs, 1989). Trace amounts of phaeophytins are present in all photosynthetic tissues (Porra *et al.*, 1997 and references therein) and removal of the magnesium ion from chl and chlorophyllide to form phaeophytin and phaeophorbide, respectively, is catalysed by a magnesium dechelatase enzyme. Also, many algal species contain acidic cell sap which may facilitate the loss of Mg (Brown *et al.*, 1991). Loss of the C-13^2 methylcarboxylate group was reported in senescent systems containing mutant algal strains (Schoch *et al.*, 1981; Ziegler *et al.*, 1988) and pyrophaeophorbide *a* (**X**) was described as representing the end product of chl breakdown during senescence (Ziegler *et al.*, 1988; Shioi *et al.*, 1991).

In order to obtain further information about the origin of sedimentary tetrapyrroles and their connection to the precursor chl *a* molecule Spooner *et al.* (1994a) carried out a series of experiments in which axenic batch cultures of the diatom *Phaeodactylum tricornutum* were induced to undergo senescence by the exclusion of light (similar to conditions experienced at the end of a bloom). The results showed a progressive defunctionalization of chl *a* after the exclusion of light, with a phaeophytin *a* maximum occurring before that of pyrophaeophorbide *a*, indicating that these products can be formed in the water column by the action of endogenous algal enzymes. In a sample of the same alga aged under anoxic conditions, pyrophaeophorbide *a* was the most abundant defunctionalization product, suggesting that it is either produced in larger quantities or is selectively preserved under anoxic conditions (Spooner *et al.*, 1994a). Surprisingly, the expected intermediates phaeophorbide *a* (**VII**) and pyrophaeophytin *a* (**XI**) were absent from almost all samples, suggesting that perhaps the hydrolysis of the phytyl ester and loss of the carbomethoxy group are catalysed by the same enzyme. This experiment also provided the first unambiguous identification of pyrophaeophorbide production during senescence of a non-mutant alga (see above).

Spooner *et al.* (1994b) carried out further experiments utilising the same alga and inducing senescence by osmotic shock followed by sonication. This resulted in the formation of chlorophyllide *a* and smaller amounts of phaeophytin *a*, phaeophorbide *a* and pyrophaeophorbide *a* within 3 h of lysis. This transformation time is comparable with the gut clearance rates of some zooplankton (Kiørboe *et al.*, 1982 and references therein), implying that the chl transformation observed during zooplankton herbivory may in part be due to the action of ingested endogenous algal enzymes (*cf.* Head and Harris, 1996; Stevens and Head, 1998).

Recently, a series of experiments was carried out to investigate the pigment changes when a variety of different algal species were induced to undergo senescence under a variety of conditions (e.g. oxic/anoxic; warm/cold). A common feature in the degradation of several species was the rapid conversion of chl *a* to phaeophorbide *a* followed by its successive loss and a coincident increase in the proportion of phaeophytin *a*, eventually leading to a predominance of phaeophytin *a* (Louda *et al.*,

1998). It was suggested that the early generation of phaeophorbide *a* is followed by enzymatic oxidative cleavage of the macrocycle, eventually leading to loss of phaeophorbide *a*, with the subsequent increase in phaeophytin *a* indicating that it was not a substrate for the proposed enzymatic oxygenolytic cleavage. Accompanying the expected transformation products (chlorophyllides, phaeophytins, phaeophorbides and their pyro counterparts), Louda *et al.* (1998) also describe a number of other products including the mono oxygenated allomers of chl *a* and phaeophytin *a* (13^2-hydroxychl *a*, **XII** and 13^2-hydroxyphaeophytin *a*, **XIII**), purpurin-18-phytyl ester (**XIVa**) and the tentatively assigned 13^1-oxydeoxo-chl *a*.

1.2.4. Oxidative Transformations

It is clear that the dominant mechanism in chl degradation involves Type II reactions in which the chromophore structure is lost. It is now known that this sequence involves photooxidation, resulting in cleavage of the macrocycle at the C-5 methine bridge, generating 19-formyl-1-oxo-bilanes (Louda *et al.*, 1998 and references therein). Linear tetrapyrroles have been found in higher plants, fresh water chlorophytes and marine organisms but no fragment smaller than a tetrapyrrole has so far been detected (Porra *et al.*, 1997). On the other hand, photooxidation of chl *a* in artificial seawater produces 1*H*-pyrrole-2,5-diones (maleimides; Rontani *et al.*, 1991), so it seems likely that mono-, di- and tripyrroles would occur naturally. Maleimides also occur in the sedimentary record (Grice *et al.*, 1996).

Oxidative chl transformation reactions are not, however, limited to Type II reactions. Type I oxidation reactions also take place and are usually centred on the exocyclic ring E, thus generating a range of transformation products incorporating additional oxygen atoms. It has been suggested that the production of the chl *a*-allomer by hydroxylation at C-13^2 may initiate a series of reactions leading to the formation of purpurins; this then opens up an additional diagenetic pathway leading to the production of chlorin-p_6 (**XV**) and ultimately to sedimentary aetioporphyrins (e.g. Louda *et al.*, 1998 and references therein). Other laboratory studies of Type I oxidation reactions (e.g. Woolley *et al.*, 1997, 1998) have shown that the formation of 13^2-OH pigment products actually

prevents further oxidation and that it is reaction with ground state molecular oxygen which leads to cleavage of the exocyclic ring (Woolley *et al.*, 1998; discussed in more detail in Chapters 4-7).

Recently, Naylor and Keely (1998) have identified a number of Type I oxidation products of chl in recent sediments (Lake Baikal, Russia; Loch Ness, Scotland) including 13^2-OH-phaeophytin *a* (**XIII**), purpurin-18 (**XIVb**) and purpurin-18-phytyl ester (**XIVa**). Both purpurins were also found in plankton samples trawled from the surface waters, indicating that the oxidised components are formed in the water column at the earliest stage of transformation.

1.2.5. Other Transformations

Other chl transformation products found in the sedimentary environment can not be explained by the "Treibs" - type hypothesis (Treibs, 1936; *cf.* fig. 1-1). To date the two most abundant types of these compounds are those containing an extended fused ring system, particularly chlorophyllone *a* (**XVI**), the other group being the steryl chlorin esters and related compounds (see section 1.2.6).

Chlorophyllone *a* (more precisely referred to as 13^2-hydroxychlorophyllone *a*; e.g. Ma and Dolphin, 1995) was first isolated from a short necked clam (Sakata *et al.*, 1990) and subsequently from an oyster (Watanabe *et al.*, 1993). Sakata *et al.* (1990) suggested that it is produced by an enzyme of the clam, symbiotic micro-organisms or the phytoplankton diet of the animals. This and other phaeophorbide *a*-related compounds isolated from a clam, scallop and oyster are thought to be the main antioxidants in these organisms (Watanabe *et al.*, 1993).

Of more significance geochemically, 13^2-hydroxychlorophyllone *a* has also been reported in a mixture of attached diatoms, diatoms cultured on plastic plates and in several other mono-cultured wafting diatom species (*Nannochloropusis oculata* and *Poryphyridium purupureum*) and a haptophyte (*Isochrysis galbana*); a further degradation product, chlorophyllone *a* lactone (**XVII**) was found in a number of other

species (Watanabe *et al.*, 1993; Sakata *et al.*, 1994). Chlorophyllone has been identified in the bottom sediment of a freshwater lake (Chillier *et al.*, 1993) and further studies (Harris *et al.*, 1995b) have shown it to occur in a wide variety of sedimentary settings, including a deep sea sediment core (ODP Leg 108; Hole 658; Harris and Maxwell, unpublished results). The origin and significance of 13^2-hydroxychlorophyllone *a* are discussed in more detail below in Chapter 4.

Recently, the unstable chlorin 13^2,17^3-cylophaeophorbide *a* enol (**XVIII**) has been found to be among the most abundant pigments in recent sediments (Black Sea, Mediterranean Sea, Peru margin) and sediment traps (Peru margin; Ocampo *et al.*, 1999). This compound is thought to be the precursor of certain other sedimentary chlorins and porphyrins containing exocyclic seven or seven and five membered rings, including 13^2-hydroxychlorophyllone *a* (e.g. Watanabe *et al.*, 1993; Ocampo *et al.*, 1999).

There are many reports of other chlorins (described as "unknown" or "unidentified") whose origins in terms of chl transformations remain unknown (e.g. Downs, 1989; Nelson, 1989; Strom, 1993). While the structures of some of these are probably unknown it seems likely, however, that some are known transformation products but were unassigned as the authors did not have access to LC-MS.

1.2.6. Steryl Chlorin Esters

1.2.6.1. Occurrence

Prowse & Maxwell (1991) discovered steryl chlorin esters (SCEs: chl biotransformation products in which the chl *a* biotransformation product, pyrophaeophorbide *a* [**X**], is esterified to a sterol) in an immature lacustrine sediment of Miocene age (Maraú Oil Shale, Brazil). The major esterified tetrapyrrole was pyrophaeophorbide *a* esterified to 24-ethyl-4α-methyl-5α(H)-cholestan-3β-ol (fig. 1-2). Its structure and that of a similar compound comprising the meso counterpart (**XIX**) of pyrophaeophorbide *a* esterified to the same stanol were assigned by FAB-MS and ^{1}H NMR and by GC-MS analysis of the sterol liberated by hydrolysis.

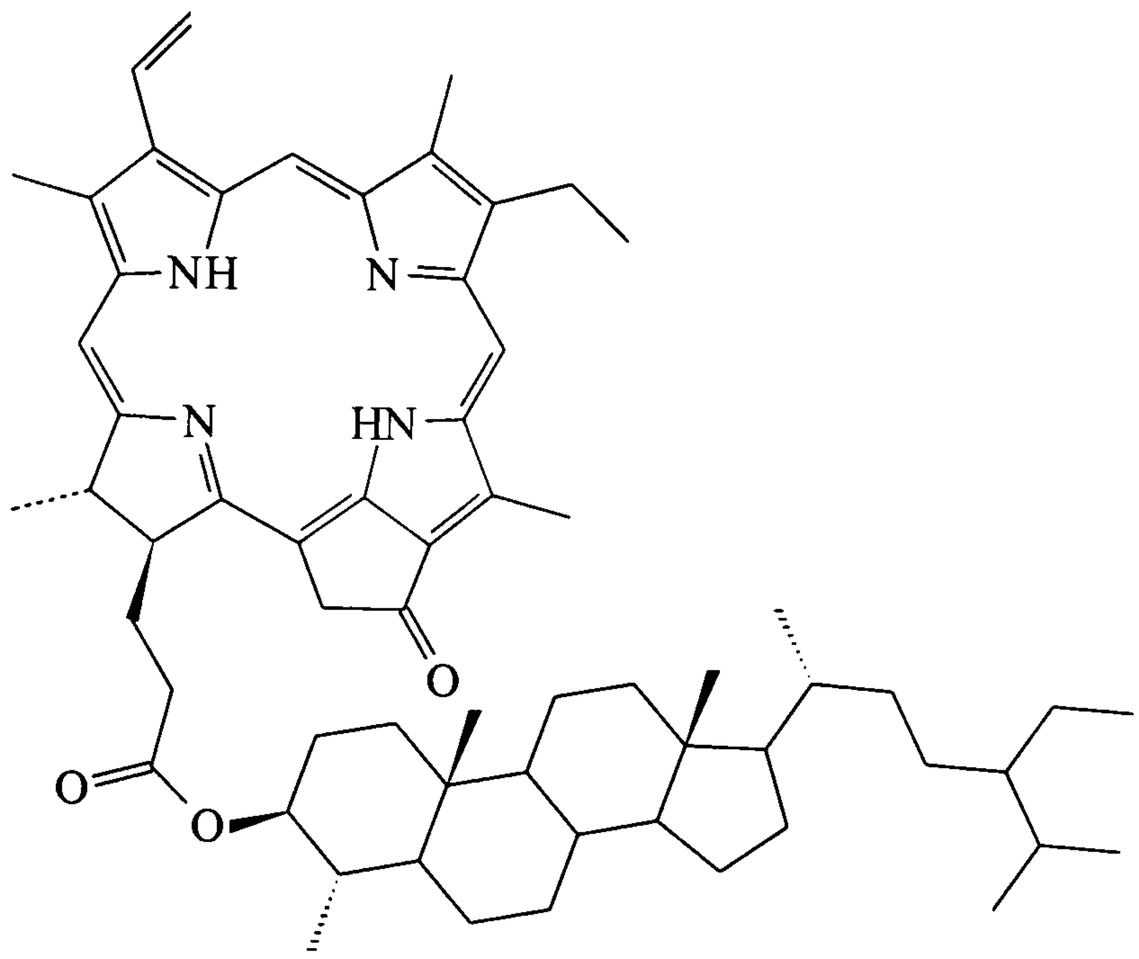


Figure 1-2. *24-ethyl-4α-methyl-5α(H)-cholestan-3β-yl pyrophaeophorbide a (Prowse and Maxwell, 1991).*

Subsequently, Eckardt *et al.* (1991b) detected a complex suite of SCEs containing a series of C_{27}-C_{30} sterols and stanols in the bottom sediment of a eutrophic lake (Priest Pot, Cumbria, UK). This suggested that the esterification of pyrophaeophorbide *a* with algal sterols might be a widespread transformation pathway for chl *a* in the aquatic environment. Likewise King & Repeta (1991) reported a series of SCEs in sediment extracts from the Black Sea, which were identified by visible spectroscopy and mass spectrometry. Following these initial reports, SCEs have subsequently been found in a wide variety of ancient and modern marine and lacustrine environments (Eckardt *et al.* 1992; Pearce *et al.*, 1993, 1998; King and Repeta, 1994; Kowalewska, 1994; Harris *et al.*, 1995a; Harradine *et al.*, 1996a; Laurelliard *et al.*, 1997; Cariou-Le Gall *et al.*, 1998; Naylor and Keely, 1998). Eckardt *et al.* (1992) concluded that, given the widespread occurrence of SCEs their formation appears to be ubiquitous on a global scale in both marine and lacustrine environments and, given their presence in the Clarkia mudstone (Early Miocene) and in another Miocene lake sediment (Prowse and Maxwell, 1991), this process has been occurring for at least the last 17 million years.

Reports of their abundance in surface sediments tend to range between 8 and 40% of the total extractable chlorins (e.g. King and Repeta, 1991; Eckardt *et al.*, 1992), so SCEs clearly represent a significant sink for the chlorophyll *a* biosynthesised in the euphotic zone of the water column.

1.2.6.2. Mode of Formation

Prowse and Maxwell (1991) suggested that the esterification occurred either during biosynthesis of an unknown precursor chlorophyll or resulted from esterification of a hydrolysis product from a known chl (chl *a*) after cellular disruption. They proposed, *a priori*, three possible pathways for the production of these novel compounds:

(i) A chemical reaction occurring in the sediment during diagenesis; however, due to the high degree of specificity of the reaction (only one esterifying alcohol was found) this was considered unlikely.

(ii) Formation during biosynthesis of an unknown chl as esterification of the propionic acid side chain is the final step in chlorophyll biosynthesis.

(iii) A biologically mediated esterification occurring between a chl derivative such as pyrophaeophorbide *a* and the sterol.

The third possibility was considered to be the most likely mode of formation and was assumed to occur after cellular disruption brought about by senescence, decay or herbivory.

Three similar modes of formation were also proposed by King and Repeta (1991) for the SCEs detected in The Black Sea :

(i) Direct biosynthesis.

(ii) Predepositional esterification or transesterification of pyrophaeophorbide *a* or pyrophaeophytin *a* with sterols during grazing or microbial degradation.

(iii) Post-depositional esterification reactions in sediments.

However, predepositional formation, probably enzymaticaly mediated by zooplankton during herbivory, was thought to be the most likely method of production. This was supported by several lines of evidence:

(i) The presence of suspected SCEs in suspended particulate matter and sediment trap material (King and Repeta, 1991 and references therein).

(ii) Differences between the distributions of 4-desmethylsterols in the SCEs and the free sedimentary sterols.

(iii) The low abundance of stanols and 4-methyl sterols in the SCE fraction.

(iv) The specificity of the nucleus of the esterified pigments i.e. only pyrophaeophorbide *a*.

To obtain more information about the mode of production of the sedimentary components, Eckardt *et al.* (1992) examined a surface plankton sample collected during a diatom bloom in the Baltic sea (Gotland Deep). The sample contained mainly phytoplankton with zooplankton (comprising mainly copepods) being scarce. The major chlorins in the sample were phaeophorbide *a* (**VII**) and phaeophytin *a* (**VIII**); their pyro counterparts (**X** and **XI** respectively) and SCEs were also detected. The SCE distribution was dominated by components containing C_{28} mono or di-unsaturated sterol moieties indicative of a sterol input from diatoms (*cf.* Volkman, 1986). The occurrence of SCEs in the bloom provided evidence that SCE formation occurs in the water column rather than in the sediment after deposition and that they may be formed during phytoplankton senescence or decay as a result of enzymatic esterification after cellular disruption (but see below). They suggested that this origin would not exclude the presence of SCEs in sediment trap material or faecal pellets if a significant proportion of the material consisted of senescent dead cells which were consumed by the zooplankton (*cf.* King and Repeta, 1991 and references therein).

King and Repeta (1994) carried out further studies of water column particulate samples, sediment trap material and sedimentary material and found that SCEs, as well as being present in the sediment, were also present in the sediment traps but were absent from the suspended particulate matter (spm). Comparison of the SCE sterols and the free sterols present in the spm showed the closest correlation, indicating that SCEs are derived from sterols present in the spm. Based on this correlation and consideration of differences between free and SCE sedimentary sterol fractions (discussed in more detail below; section 1.2.6.3.) and an earlier report of chl degradation products suspected to be SCEs in faecal pellets (King and Repeta, 1991 and references therein) they concluded that SCEs are formed by enzymatic esterification during zooplankton herbivory.

A senescence origin was also not supported by the work of Pearce (1994) who did not detect SCEs when the diatom *Phaeodactylum tricornutum* (a species known to contain high abundances of the enzyme chlorophyllase) was induced to undergo senescence; also there have not been any other reports of SCEs in similar experiments. An origin from zooplankton herbivory therefore seemed the most likely option. This hypothesis

was also supported indirectly by the fact that the only sedimentary environment examined for the presence of SCEs which failed to yield such components is a karstic lake environment (Lake Ciso, Spain) which does not contain any zooplankton (Villanueva *et al.*, 1994).

To test the herbivory hypothesis a small scale feeding experiment in which the copepod *Calanus helgolandicus* was allowed to graze on the diatom *Thalassiosira weissflogii* for 48 hours was carried out (Harradine *et al.*, 1996b). Analysis of the pigments in a mixture of remaining alga and faecal pellets and also of pellets which had been aged in the dark for 35 days confirmed the presence of SCEs in both the fresh and aged samples. The original algal culture and an algal control run alongside the experiment confirmed the absence of SCEs in the source organism. Comparison of the culture free sterol distribution and the SCE sterol distribution showed a close correlation between the two fractions, suggesting that the enzymatic esterification/transesterification reaction did not appear to discriminate amongst sterols. The cholesterol (**A1**) ester of pyrophaeophorbide *a* in the pellets was produced from sterols originating from the animal alone as no cholesterol was detected in the algal culture (cholesterol is commonly the most abundant sterol in zooplankton and their faecal pellets; Gagosian *et al.*, 1981; Corner *et al.*, 1986). When cholesterol was excluded from the comparison it was found that the SCE sterol distribution resembled that of the algal culture more closely as suggested by Repeta (1995) who also proposed that cholesterol should be omitted from comparisons of SCE and free sterol distributions even though cholesterol has been reported in some algal species, sometimes being the dominant sterol (e.g. Volkman, 1986). The presence of SCEs in faecal pellets in this experiment provided the first direct evidence that herbivory was responsible for the accumulation of significant concentrations of SCEs in sediments due to the efficient method of transport of these compounds in large, often aggregated, faecal pellet material which has been shown to contribute significantly to the downward flux of organic carbon in oceans (e.g. Welschmeyer and Lorenzen, 1985).

Further evidence for SCE production during grazing was shown by King and Wakeham (1996), who detected them in the gut contents and faecal pellets of Salps (gelatinous macrozooplankton) from the Sargasso Sea. Salps have been shown to produce faecal

pellets within 16 h of feeding and these may play an important role in transferring organic matter from surface waters to sediments. SCEs were detected in high abundance in the guts but were absent from samples of suspended particulate matter from the same region, the food source for the salps. Close correlation was shown between the free sterols in the suspended particulate matter, the gut free sterols and the gut SCE sterols.

1.2.6.3. Potential Significance

The early studies (King and Repeta, 1991; Eckardt *et al.*, 1992) demonstrated that there was at least a qualitative relationship between the SCE sterol distribution and the source organisms. If this was the case and no selective degradation of individual SCEs occurred then, as suggested by King and Repeta (1994), sedimentary SCEs could be used as palaeooceanographic tracers of phytoplankton species composition.

To further investigate this relationship King and Repeta (1994) looked at the distributions of SCEs and non-esterified, solvent extractable sterols (free sterols) in suspended particulate matter, sediment traps, and sediments from the Black Sea. Comparison of the sterols in the upper water column and the sedimentary SCE sterol distribution revealed good correlation, including the stanol/stenol ratios, indicating that SCEs are derived directly from sterols present in water column particulate matter. However, relative abundance of the SCEs in the sediment traps varied over the course of the Spring and Autumn blooms, possibly resulting from changes in the heterotrophic community structure. These changes could also reflect the succession of herbivorous zooplankton in the Black Sea (Burkill *et al.*, 1987), with different zooplankton having varying abilities (or perhaps some lacking the ability) to metabolise chl *a* to SCEs. Furthermore, in feeding experiments using a number of different species of zooplankton, Downs (1989) earlier identified chl degradation products (later suspected to be SCEs; King and Repeta, 1991) in the faecal pellets of certain euphausiids, *Pleuroncodes* crabs, *Phialidium* medusea, *Pleurobrachia*, and calanoid copepods but these products were produced in differing quantities depending on both herbivore and substrate.

King and Repeta (1994) suggested that if the SCE sterol distribution is preserved during sedimentation and burial, then changes in phytoplankton community structure should be recorded in the sedimentary SCE sterol record. To test this hypothesis they examined down core changes in the SCE sterol distribution at a site in the Black Sea by looking at samples from three different sedimentary units, each representing deposition under different environmental conditions. Significant differences in the distributions were apparent and were particularly evident in the abundance of the 24-ethylcholest-5-en-3β-ol (**A8**) and 4α,23,24-trimethylcholestan-3β-ol (dinosterol, **B10**) esters of pyrophaeophorbide *a*. The high relative abundance of the dinosteryl ester in the second sedimentary unit suggested that dinoflagellates were major producers in the Black Sea at time of Unit II deposition relative to Units I and III. The sediment free sterols had different distributions to both the sediment SCE sterols and the sediment trap free sterols, the most striking differences being the high amounts of 4-methyl sterols and high stanol/stenol ratios in the free sterols relative to SCEs. These differences were explained by the greater preservation of 4-methyl sterols relative to 4-desmethyl sterols during biodegradation (Gagosian *et al.*, 1980; Wolff *et al.*, 1986; Harvey *et al.*, 1989) and the known increase in stanol/stenol ratios during biodegradation (*cf.* Gagosian and Heinzer, 1979; Teece, 1994). The slower degradation of 4-methyl sterols was clearly supported, as 4-desmethyl sterols accounted for 98% of the free sterol flux but only 20% of the free sterols in the sediments, the balance in each case coming from 4-methyl sterol input. Therefore, it was suggested that the esterification to pyrophaeophorbide *a* may protect 4-desmethyl sterols from selective degradation as 99% of the sedimentary SCE sterols were 4-desmethyl sterols and only 1% were 4-methyl sterols. Likewise, it was suggested that the diagenetic reduction of stenols to stanols was retarded in the esters as suggested by Cranwell and Volkman (1981).

The free and SCE sterol distributions from five other sites (Baltic Sea, Black Sea, ODP Leg 108 Site 658C, Lake Valencia and Priest Pot) have also been examined (Pearce *et al.*, 1998) and again significant differences in the 4-methyl sterol and stanol/stenol ratios, comparable with those found by King and Repeta (1994), were evident in both sterol fractions from all but one site. In the latter case (Priest Pot) the abundance of 4-methyl sterols in both the free and esterified fractions showed no significant difference

and it was suggested that this could be due to the shallow water column leading to resuspension. It was also noted that, where present, C_{26} sterols also existed in higher abundance as SCEs than as free sterols, suggesting that the free C_{26} sterols are more susceptible to biodegradation than their higher molecular weight counterparts.

In summary, there is field evidence indicating that sedimentary SCE sterols appear to represent more accurately than free sedimentary sterols the sterol distribution of the original phytoplanktonic community (King and Repeta, 1994; Pearce *et al.*, 1998).

1.2.6.4. Related Products

Alternative chlorin moieties

It is clear that the most abundant chlorin esters detected to date are components in which algal and zooplankton derived sterols are esterified to pyrophaeophorbide *a* (**X**). However, other high molecular weight chlorin esters have also been detected, such as the mesopyrophaeophorbide *a* (**XIX**) ester of the C_{30} stanol mentioned above (Prowse and Maxwell, 1991) and a preliminary result indicating the presence of pyrophaeophorbide *b* (**XX**) in low abundance in the hydrolysis products of an SCE fraction from Lake Valencia, Venezuela (Pearce *et al.*, 1993). Subsequently, Cariou-Le Gall *et al.* (1998) found SCEs (SCEs *b*) in which pyrophaeophorbide *b* was esterified to the two most abundant SCE *a* sterols within the pyrophaeophorbide *a* fraction in Mediterranean sapropel samples. This was the first direct observation of these chl *b* transformation products using both uv/vis spectrophotometric and mass spectrometric methods (*cf.* Pearce *et al.*, 1993). Since the two SCEs *b* occurred in the same relative abundance as their SCE *a* counterparts this study suggested that chl *b* undergoes the same transformation as chl *a*. Recently Riffé-Chalard *et al.* (1999) have reported the occurrence of phaeophorbide *a* (**VII**) steryl esters in an oxic lake sediment. This represents the first identification of such components in the natural environment although related components have been reported (see below). It should also be noted that the formation of phaeophorbide *a* esters has been demonstrated in the laboratory where it was shown that both pyrophaeophorbide *a* and phaeophorbide *a* react to form esters equal efficiency (Pearce, 1994).

Alternative esterifying alcohols

Analysis of the high molecular weight chlorin fraction from Lake Valencia sediment revealed the presence of two components eluting just after the SCEs and whose mass spectra suggested they were triterpenoid alcohols esterified to pyrophaeophorbide *a* (Harradine *et al.*, 1996a). Since cyanobacteria are present in high abundance in the lake and are known to biosynthesise hopanoids such as bacteriohopanetetrol (**XXI**) as cell membrane rigidifiers (Ourisson *et al.*, 1979), it was suggested that these components could be hopanoid chlorin esters. The first component was identified as the (22*R*)-30a,30b-dihomohopan-30b-ol (**XXII**) ester of pyrophaeophorbide *a* by synthesis of a standard which was co-injected with the original sediment extract. The second was assigned as the tetrahymanyl ester (**XXIII**) by co-injection of tetrahymanol with the reduction products ($LiAlH_4$) of the sediment fraction. Tetrahymanol is of ciliate origin and occurs widely in marine and lacustrine sediments (e.g. ten Haven *et al.*, 1989, Venkatesan, 1989; Sinninghe-Damsté *et al.*, 1995). Ciliates only synthesise tetrahymanol when there is a shortage in the supply of dietary sterols (Harvey and McManus, 1991). It is unclear, however, if the tetrahymanol ester is produced by ciliate grazing or during zooplankton grazing on a mixed diet of ciliates and phytoplankton.

Laureillared *et al.* (1997) recently reported long chain mono-unsaturated and saturated *n*-alcohol chlorin esters in sediment samples from the Indian ocean. However, the alcohols were only detected after hydrolysis of the SCE fraction following TLC purification to remove any contaminating free alcohols; clearly confirmation is required that these *n*-alcohols occur as chlorin esters.

A novel series of components in which certain degradation products (isofucoxanthin-dehydrate, **XXIV**; isofucoxanthinol-dehydrate, **XXV**) of the carotenoid fucoxanthin (**XXVI**) are esterified to phaeophorbide *a* (**VII**) and pyrophaeophorbide *a* (**X**) have been found in a number of recent marine and lacustrine sediments (Goericke *et al.*, 1999). These carotenol chlorin esters (CCEs) were found to account for between 20 and 40% of the total chlorins, clearly representing an important transformation pathway for both chl *a* and fucoxanthin in this environment. It was proposed that they are derived from a similar process occurring in the guts of herbivorous zooplankton that produces SCEs.

As fucoxanthin is a primary carotenoid component of diatoms it was also suggested that these CCEs are uniquely derived from grazing on diatoms and could serve as biomarkers for this process.

1.2.6.5. Summary

SCEs and related esterified chlorin components are abundant chl biotransformation products found to occur almost ubiquitously in the aquatic sedimentary environment and represent a significant sink for the chlorophyll biosynthesised in the euphotic zone of the water column. Comparison of free and sedimentary SCE sterol distributions highlights significant differences in the two fractions and the SCE distribution is thought to be a better indicator of phytoplankton community structure. To further establish this hypothesis it is necessary to investigate in more detail the relationship between the sterols of specific algal substrates and the SCE distributions produced from them.

1.3. PRESENT STUDY

The aim of this work was to investigate further the extent of production of SCEs and their relationship to substrate organisms, as well as to examine changes in SCE abundance and distribution during ageing of faecal pellets by performing feeding experiments (*cf.* preliminary study of Harradine *et al.*, 1996b). The study is summarised as follows:

- A series of feeding experiments was performed by the author in conjunction with Course, Head and Harris (Plymouth Marine Laboratory) in which the copepod *Calanus helgolandicus* was allowed to graze on a variety of different algal species chosen to represent the main algal classes. The chapters (2-5) describing these experiments are presented in order of increasing complexity of the results rather than in the order in which they were performed (Table 1-1).

	Species	Algal Class
Chapter 2		
	Tetraselmis suecica	Prasinophyte
	Dunaliella tertiolecta	Chlorophyte
	Chlamydomonas reginae	Chlorophyte
Chapter 3		
	Isochrysis galbana	Haptophyte
	Coccolithus pelagicus	Haptophyte
	Pleurochrysis carterae	Haptophyte
Chapter 4		
	Thalassiosira weissflogii	Diatom
Chapter 5		
	Prorocentrum micans	Dinoflagellate
	Alexandrium tamarensis	Dinoflagellate

Table 1-1. *Algal species used in copepod feeding experiments.*

- Other possible sources of SCE production were investigated *via* herbivory experiments with the marine heterotrophic dinoflagellate *Oxyrrhis marina* grazing on *Isochrysis galbana* and a green alga and with a mixed community of small mesozooplankton grazing on the cyanobacterium *Synechococcus* sp. (Chapter 6).

Chapter 2

FEEDING EXPERIMENTS WITH GREEN ALGAE

2.1. INTRODUCTION

2.1.1. Previous Work

Harradine *et al.* (1996b) demonstrated the production of SCEs during zooplankton herbivory *via* a feeding experiment in which the copepod *Calanus helgolandicus* was allowed to graze on the diatom *Thlassiosira weissflogii*. Examination of the pigment signature (by HPLC-PDA-APCI-MS) of the faecal pellets revealed at least four SCE components in which the chl *a* (**I**) transformation product pyrophaeophorbide *a* (**X**) was esterified to mono- or di-unsaturated sterols. This work represented the first conclusive identification of SCEs within faecal pellets of copepods although earlier experiments of Downs (1989), demonstrated the production of non-polar chlorophyll degradation products later suspected to be SCEs (King and Repeta, 1991, 1994).

2.1.2. Diversity of Production of SCEs and Relationship to Substrate

The formation of SCEs has only been demonstrated conclusively in the laboratory with one herbivore and one algal species although King and Wakeham (1996) found SCEs in the guts and faecal pellets of salps grazing naturally. Therefore to determine more precisely the relationship between precursors (algal chl and sterols, and animal sterols) and product (SCEs) it is necessary to carry out further feeding experiments with a variety of different zooplankton and algal species in order to determine which herbivores are capable of this transformation and to assess which classes of phytoplankton are suitable substrates. Such experiments should also highlight any factors which result in differences in the SCE sterol distribution relative to the substrate sterol distribution such as that proposed by Repeta (1995) with regard to input of animal cholest-5-en-3β-ol.

2.1. PRESENT STUDY

2.2.1. Background

Pyrophaeophorbide *a* (**X**) is by far the most abundant SCE nucleus of the high molecular weight pigment fractions in sediments, with only three comparatively isolated exceptions being known. Prowse and Maxwell (1991) found minor amounts of meso-pyrophaeophorbide *a* (**XIX**) esterified to a stanol in the Miocene Maraú oil Shale (Bahia State, Brazil). Pyrophaeophorbide *b* (**XX**) was present in low abundance relative to pyrophaeophorbide *a* in the hydrolysis product of an SCE fraction from Lake Valencia (Venezuela), although the parent SCEs were not apparent in the SCE fraction itself (Pearce *et al.*, 1993). Also, two recent reports have demonstrated the existence of phaeophorbide *a* (**VII**) steryl esters (Riffé-Chalard *et al.*, 1999) and carotenol esters (CCEs; Goericke *et al.*, 1999) as well as their pyrophaeophorbide *a* counterparts in a number of marine and lacustrine settings.

During the course of this work two further reports of the occurrence of sedimentary SCEs containing the pyrophaeophorbide *b* moiety (SCEs *b*) have come to light, suggesting that SCEs may also be an important sink for chl *b* as well as chl *a*. Cariou-Le Gall *et al.* (1998) detected two SCEs *b* in Mediterranean sapropel samples (ODP leg 160, Holes 966D and 969C) and recently Kowalewska *et al.* (1999) reported minor quantities of SCEs *b* in sediments from the Gotland Deep (Baltic Sea). Although it is assumed that SCEs *b* are also produced by biologically mediated esterification during herbivory, their formation has not been demonstrated directly in the laboratory and is therefore a focus of this work.

2.2.2. Occurrence of Chlorophyll *b*

Chlorophyll *b* (**II**) is the second most abundant chl in natural biomasst. It is distinguished from chl *a* (**I**) by the presence of 7-formyl instead of a 7-methyl substituent. This substitution shifts the red absorption band to shorter wavelengths and the Soret band to longer wavelengths, thus extending the range of wavelengths which

can be utilised from either side of the visible spectral region. Chlorophyll *b* occurs in higher plants, ferns, mosses and the eukaryotic green algae including the Chlorophyta and Euglenophyta and in some prokaryotic Prochlorophyta (e.g. Svec, 1991; Jeffrey and Vesk, 1997). It has also recently been identified along with a chl *c*-type pigment in a few Chromophytes e.g. *Mantionella squamata* (Scheer, 1991 and references therein). Where chl *b* is present the ratio of chl *a* to chl *b* is usually 2-3:1 (e.g. Svec, 1991).

The division Chlorophyta contains the two main classes of green microalgae, the Chlorophyceae (chlorophytes; 350 genera and 2500 living species) and the Prasinophyceae (prasinophytes; 13 genera and 120 living species; Jeffrey and Vesk, 1997). The Chlorophyta have a wide geographical range, being common in freshwater habitats and marine systems in both coastal waters and in the open ocean (e.g. Jeffrey, 1976; Jeffrey and Hallegraeff, 1980a, 1980b) and hence are important constituents in present-day marine ecosystems. Chlorophytes and prasinophytes are both represented in all phytoplankton size classes from microplankton (20-200 μm) to nanoplankton (2-20 μm) and picoplankton (0.2-2 μm). Prasinophytes are thought to be the more primitive forms in the Chlorophyta (Mattox and Stewart, 1984) and can have significantly different lipid distributions from those of the chlorophytes.

In addition to chl *a* and *b*, the Chlorophyta also contain a range of carotenoids. Some species of Prasinophyceae (e.g. *Tetraselmis*) contain identical carotenoids to those found in Chlorophyceae, whilst others contain significantly different distributions. Other species (e.g. *Pycnococcus provasolii*) have been found to contain magnesium-2,4-divinyl pheoporphyrin a_5 monomethyl ester (MgDVP, **XXVII**) as well as chls *a* and *b*.

2.2.3. Feeding Experiments

A series of herbivory experiments involving starved Stage V and females of *C. helgolandicus* grazing on individual cultures of *Tetraselmis suecica* (prasinophyte), *Dunaliella tertiolecta* (chlorophyte) and *Chlamydomonas reginae* (chlorophyte), were performed at Plymouth Marine Laboratory. This zooplankton species was chosen as it is the dominant species of large mesozooplankton present in the North Atlantic. It is

considered to be representative of copepods and its size, abundance and production of discreetly packaged faecal material (faecal pellets) makes it a convenient organism for use in feeding studies (Harris, personal communication). The algal species chosen, particularly *T. suecica* and *D. tertiolecta*, are frequently used as model representative species of prasinophytes and chlorophytes (Harris, personal communication). The prasinophyte *T. suecica* (size 6-15 μm) is a common northern coastal species, originally isolated from La Spezia in the Mediterranean. The chlorophyte *D. tertiolecta* (size 12-15 x 9-13 μm) is a common species in Atlantic coastal areas and was originally isolated from Oslofjord, Norway. *C. reginae* is also an Atlantic coastal species originally isolated from Roscoff, France.

The investigation of SCEs *b* production took place in two stages. Stage 1 involved three small scale feeding experiments with *C. helgolandicus* grazing on anexic cultures of the three above species in order to test for the potential to produce SCEs, with the option to repeat one of the experiments on a larger scale if the results were inconclusive.

The faecal pellets after 48h in the small scale experiments revealed the presence of SCEs *a* but SCE *b* data were inconclusive (see below). Therefore, Stage 2 involved repeating the *T. suecica* experiment on a significantly larger scale (x 5). All of the pellets were collected and, along with samples of the algal culture and an algal control, were characterised by HPLC-PDA-APCI-MS (fig. 2-1).

It should be noted that the electronic data for the Stage 1 experiments were lost due to a hard disk failure so only a brief discussion of the pigment base peak chromatograms and mass spectra of the most significant components are given for the Stage 1 results and a full discussion of the pigment assignments is given for the Stage 2 results (section 2.4.) .

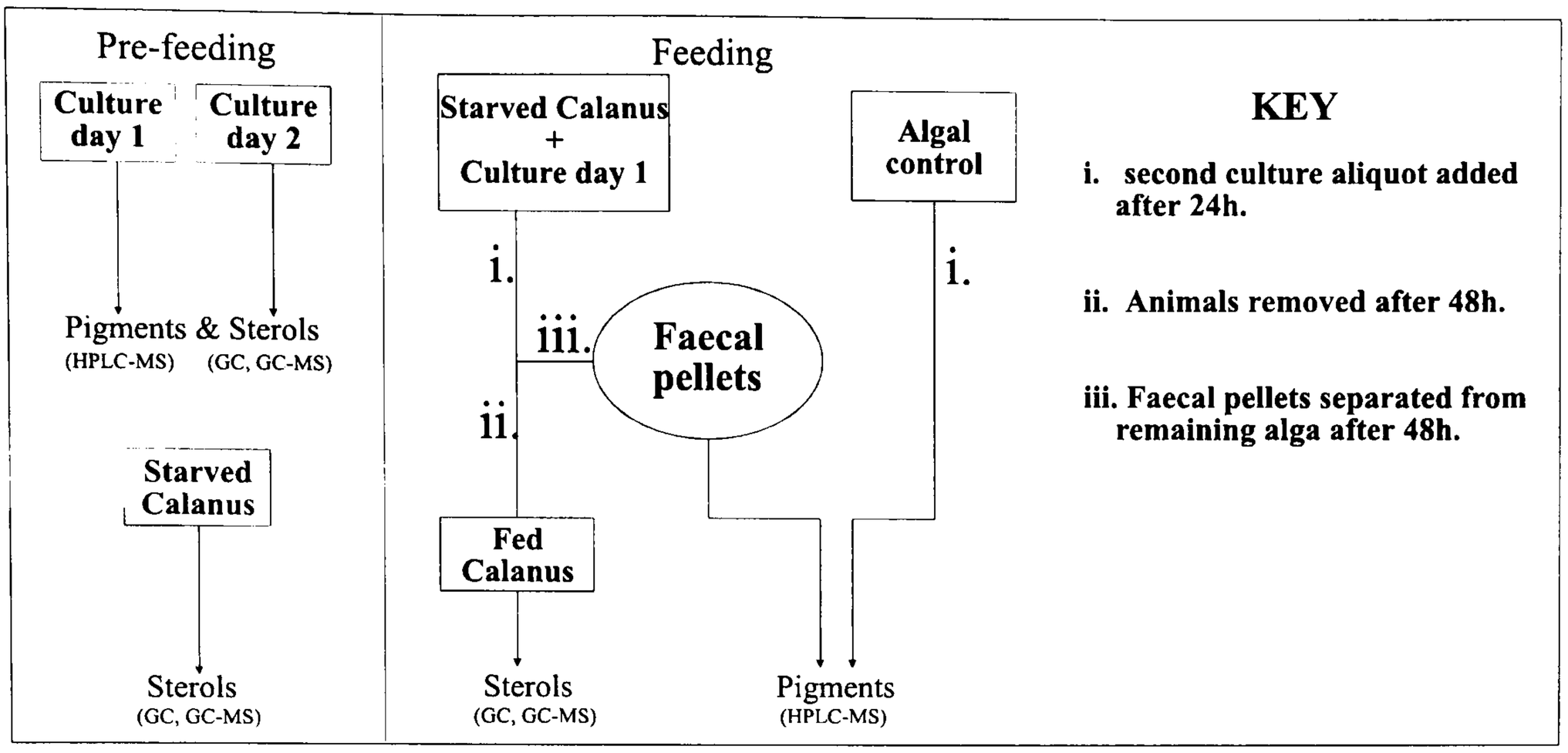


Figure 2-1. *Outline of samples and analysis protocols for Stage 2 large scale C. helgolandicus and T. suecica feeding experiment.*

2.3. STAGE 1: RESULTS AND DISCUSSION

2.3.1. Algal Cultures

LC-MS of the three algal extracts (figs. 2-2, 2-3, 2-4) shows that both chl *a* (peak 4, **I**) and its C-13^2-(*S*) epimer (4') are abundant components.

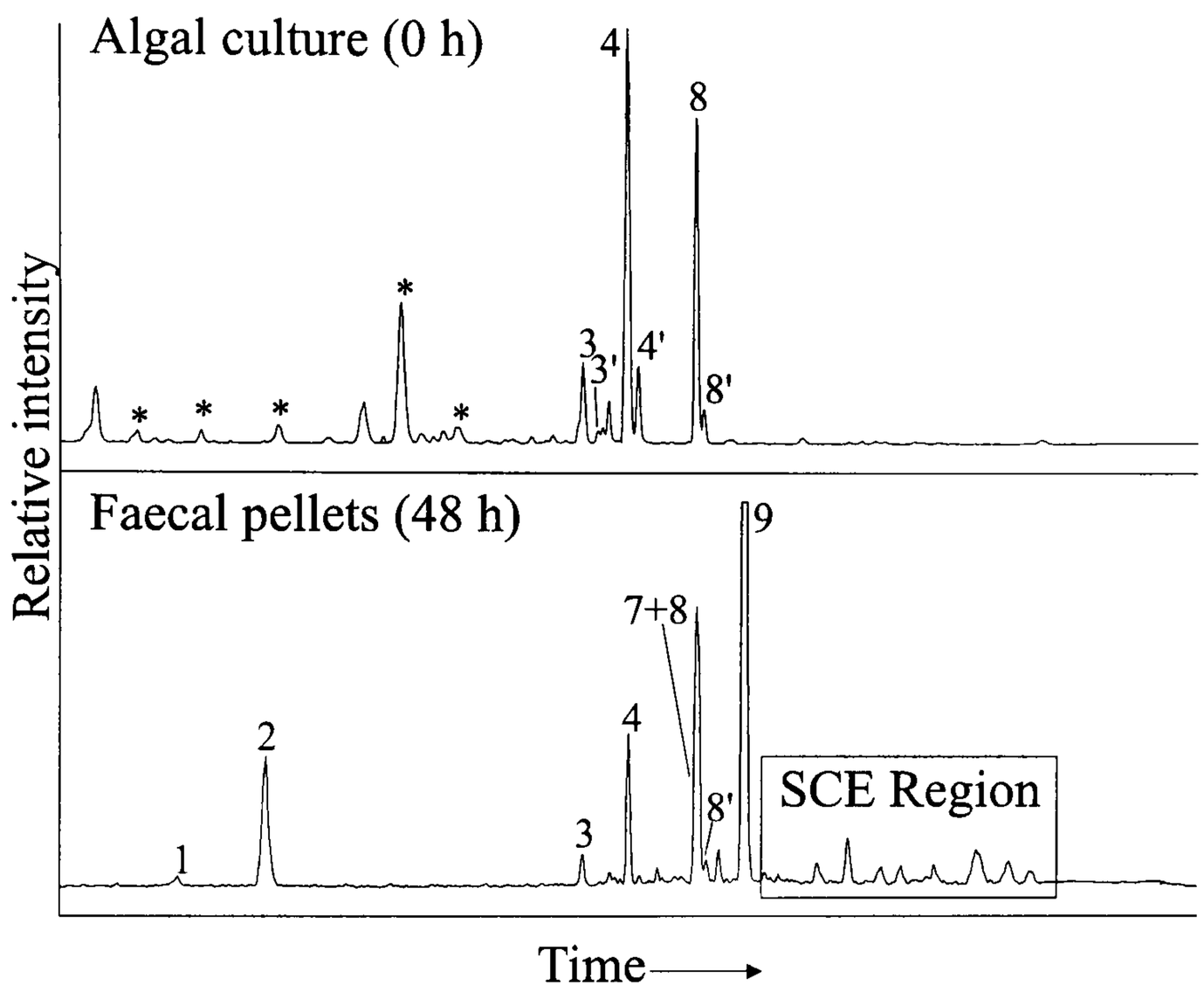


Figure 2-2. *LC-MS base peak traces from copepod feeding on Dunaliella tertiolecta.*

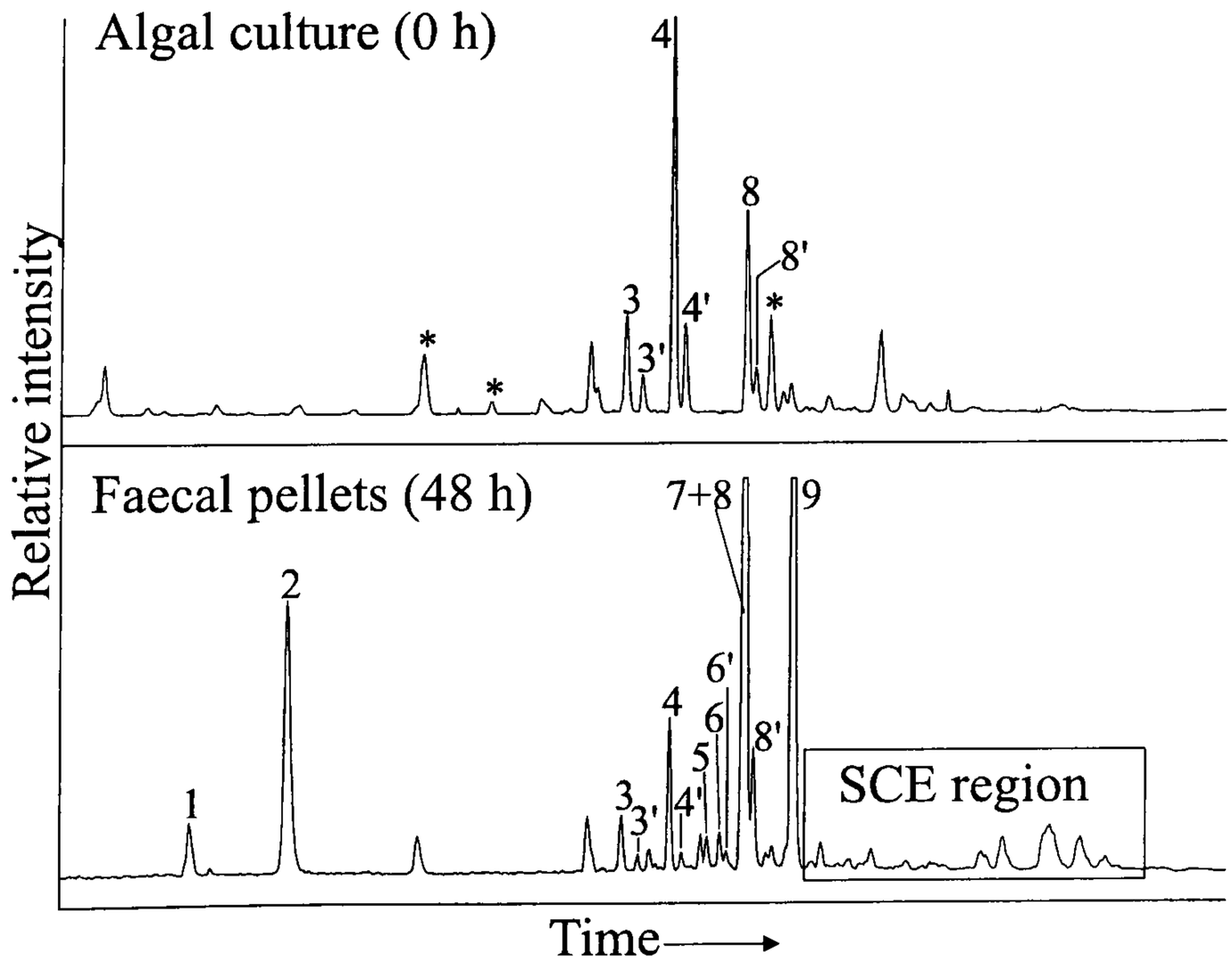


Figure 2-3. *LC-MS base peak traces from copepod feeding on Chlamydomonas reginae.*

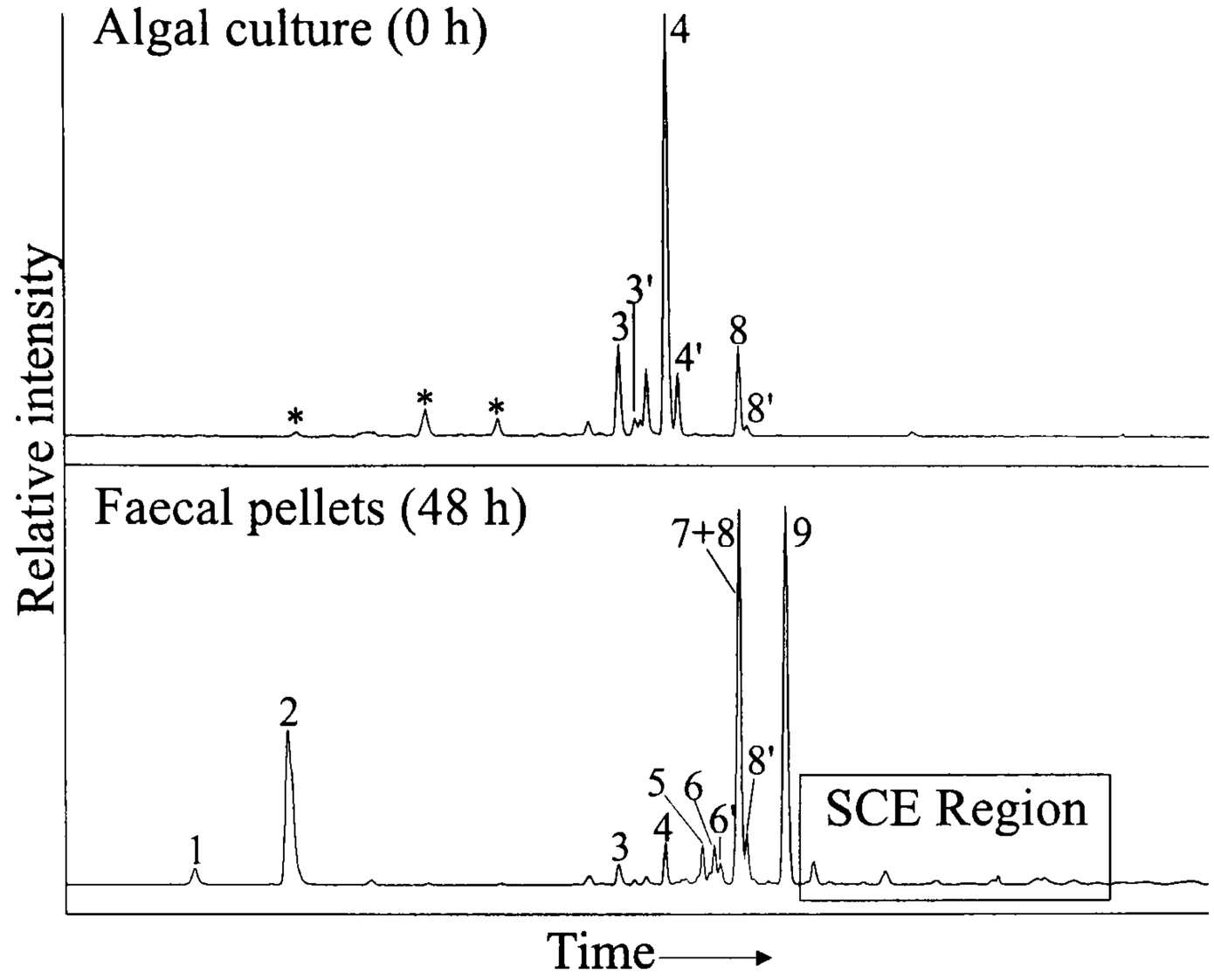


Figure 2-4. *LC-MS base peak traces from copepod feeding on Tetraselmis suecica.*

The mass spectrum of chl *a* (fig. 2-5a) shows MH^+ at *m/z* 893 with ions at *m/z* 871 (loss of Mg and addition of 2 hydrogens), *m/z* 813 (loss of C-13^2 carbomethoxy group), *m/z* 593 (loss of $C_{20}H_{38}$, phytadiene), *m/z* 615 (Mg counterpart of *m/z* 593) and *m/z* 555 and 533 (loss of C-13^2 carbomethoxy group from *m/z* 615 and 593 respectively). Also present are ions at *m/z* 839 and 583 (loss of methanol from *m/z* 871 and 615, respectively) which were not observed by Harris *et al*. (1995a), possibly due to the use of a higher vaporiser temperature in the earlier study.

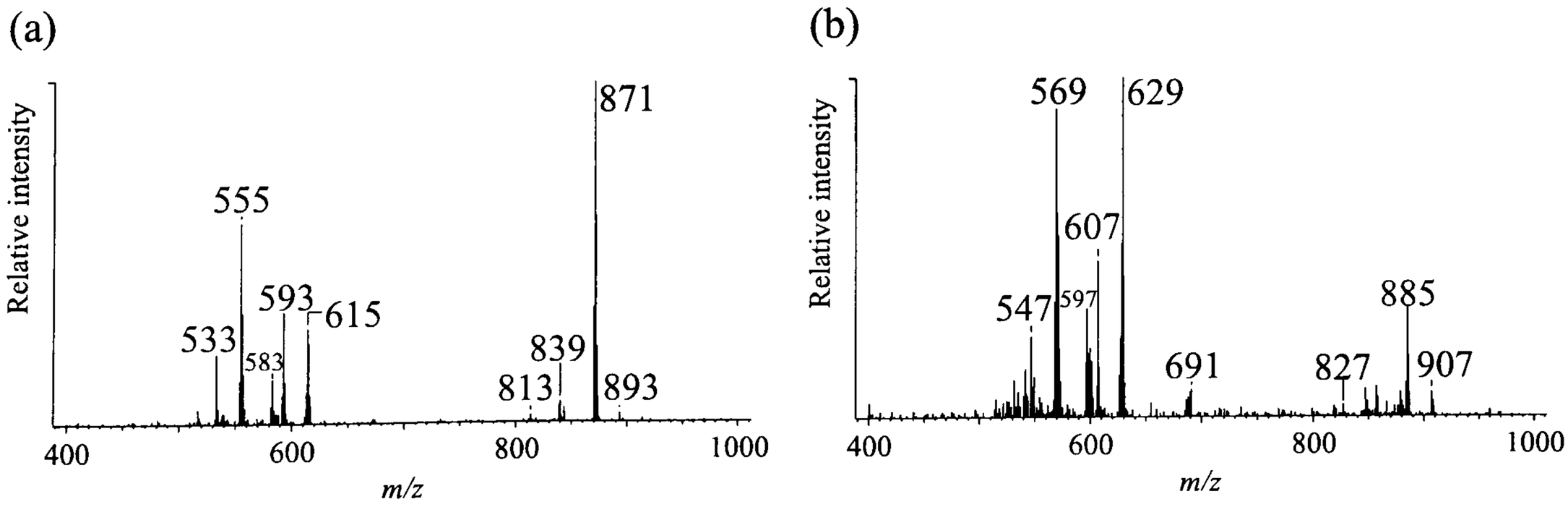


Figure 2-5 . *APCI mass spectra of (a) chl a and (b) chl b.*

Chlorophyll *a* shows loss of the C-13^2 carbomethoxy group by two different mechanisms i.e. (MH^+ - CO_2Me)+H, a formal loss of 58 mass units or MH^+ -HCO_2Me, a formal loss of 60 mass units, as previously noted for phaeophytin *a* under APCI conditions (*cf.* Harris *et al.*, 1995a). Overall, the spectrum is qualitatively similar to that observed previously with variations in the relative abundances of the ions again presumably due to the use of a lower vaporiser temperature (500°C compared to 550°C used previously; Harris *et al.*, 1995a).

Also present in all three cultures is chl *b* (peak 3, **II)** and its C-13^2-(*S*) epimer (3'). The major ions (fig. 2-5b) are analogous to those for chl *a* with a protonated molecule of *m/z* 907 and fragment ions of *m/z* 885 (loss of Mg and addition of 2 hydrogens), *m/z* 827 (loss of C-13^2 carbomethoxy group), *m/z* 607 (loss of $C_{20}H_{38}$), *m/z* 629 (Mg counterpart of *m/z* 593) and *m/z* 569 and 547 (loss of C-13^2 carbomethoxy group from *m/z* 615 and 593, respectively). The cluster of minor ions around *m/z* 691 is unassigned and may be due to co-elution of non-absorbing components. The only other significant difference to the chl *a* spectrum is the absence of an ion at *m/z* 853 corresponding to loss of methanol from *m/z* 885 (*cf. m/z* 839 in chl *a* spectrum).

The other major component in all three samples is phaeophytin *a* (8, **VIII**) partially co-eluting with its C-13^2-(*S*) epimer (8'). There was no indication of phaeophytin *b* (**XXVIII**). Also observed were a number of carotenoids (designated *, figs. 2-4, 2-5, 2-6) which were not investigated further, several peaks with no absorbance spectra and a number of peaks which could not be assigned due to a hard disk failure resulting in the loss of the electronic data.

2.3.2. Faecal Pellets

The chromatograms from all three experiments (figs. 2-2, 2-3 and 2-4) show similar distributions dominated by pyrophaeophytin *a* (9, **XI**) and pyrophaeophorbide *a* (2, **X**). The pyrophaeophytin *a* spectrum (fig. 2-6a) consists simply of the MH^+ at *m/z* 813 and a single fragment ion showing loss of $C_{20}H_{38}$ at *m/z* 535. The pyrophaeophorbide *a* spectrum (fig. 2-6b) consists of only the protonated molecule at *m/z* 535. Also evident in

all pellet samples are both C-13^2 epimers of phaeophytin *a* (8 and 8') indicating that not all of the ingested chl was converted to pyrochlorins (*cf.* Head and Harris, 1992) and minor quantities of their mono-oxygenated allomers (13^2-hydroxyphaeophytin *a*; 6 and 6', **XIII**) which are thought to be artefacts of the extraction procedure (Harradine, 1996).

The chl *b* products included pyrophaeophytin *b* (7, **XXIX**), co-eluting with phaeophytin *a* (fig. 2-7a), showing MH^+ at *m/z* 827, a major fragment at *m/z* 549 (loss of $C_{20}H_{38}$) and ions of *m/z* 799 and 521 (tentatively assigned as loss of the C-7 formyl group (-CHO) and addition of a hydrogen from *m/z* 827 and 549 respectively). Loss of the C-7 formyl group was not observed previously under negative ion thermospray conditions (Eckardt *et al.*, 1991b). Pyrophaeophorbide *b* (1, **XX**; fig. 2-7a) and phaeophytin *b* (5, **XXVIII**) were also present along with small quantities of intact chl *a* and chl *b* indicating that some material was not degraded during passage through the zooplankton gut (*cf.* Roy *et al.*, 1989).

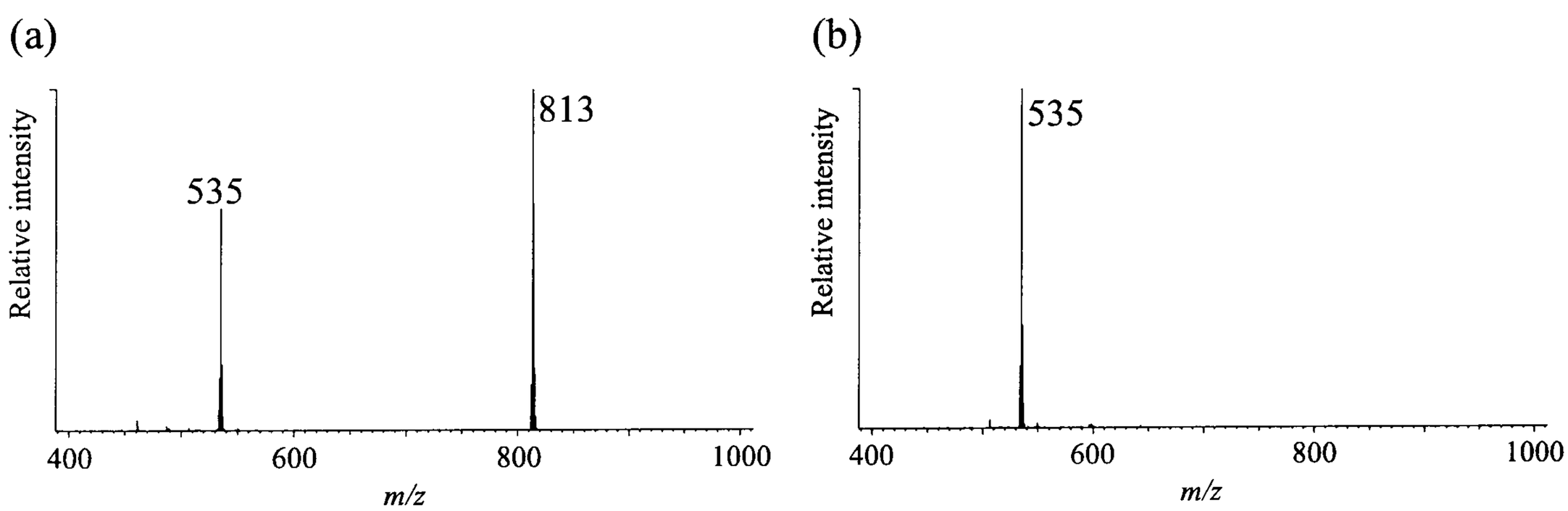


Figure 2-6 . *Mass spectra of (a) pyrophaeophytin a and (b) pyrophaeophorbide a.*

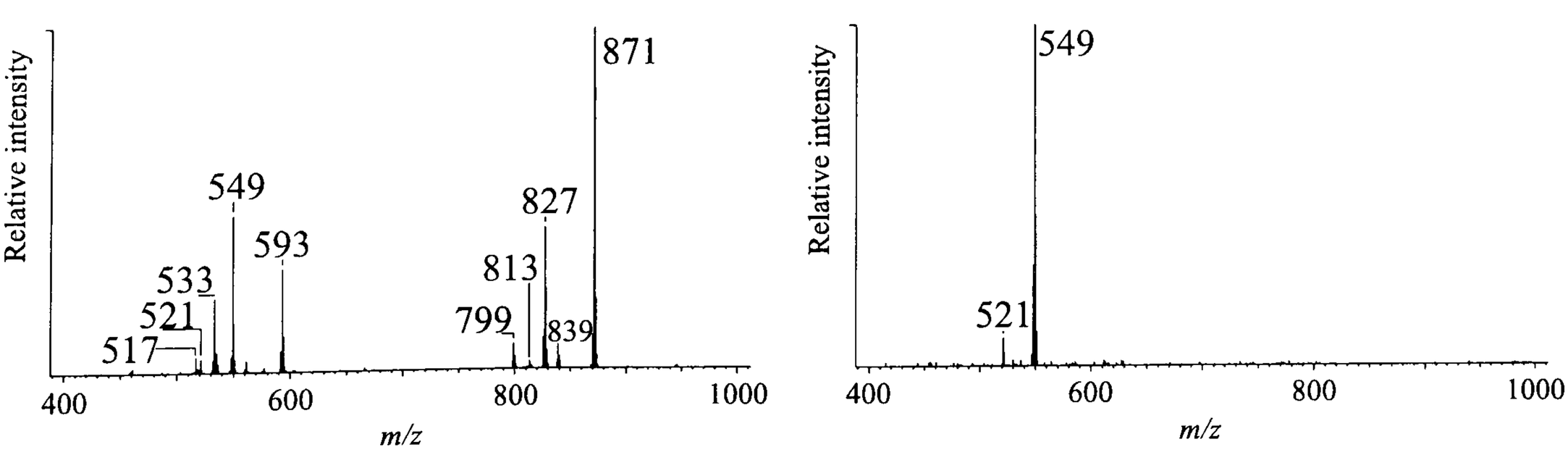


Figure 2-7 . *Mass spectra of (a) pyrophaeophytin b (co-eluting with phaeophytin a) and (b) pyrophaeophorbide b.*

Mass chromatography of each faecal pellet sample revealed the presence of SCEs *a* (e.g. *T. suecica* fig. 2-8) with MH^+ at *m/z* 901, 903, 915, 917 indicating at least four esterified sterols (mono- and diunsaturated C_{27} and C_{28} sterols). Fragment ions of *m/z* 535, coincident in t_R, indicate a pyrophaeophorbide *a* macrocycle (e.g. fig. 2-9a). Two weak *m/z* 549 peaks (fig. 2-8) between pyrophaeophytin *a* (peak 9) and the SCEs *a* suggest the presence of the pyrophaeophorbide *b* moiety (see fig. 2-9b,c); however, they show a high abundance of non-SCE derived ions indicating co-elution (*cf.* Chapter 3) and the electronic spectra were weak and inconclusive, so the production of SCEs *b* remained unconfirmed at this stage.

A group of minor peaks (fig. 2-8) with *m/z* values between 945 and 1003 eluted slightly later than the SCEs *a*. There was no indication of the *m/z* 535 pyrophaeophorbide *a* breakdown ion (e.g. fig. 2-9d) and the electronic spectra, although generally similar to those of chl *a* transformation products did show in some cases a bathochromic shift in the Soret band of between 3 and 6 nm relative to the SCEs (only the blue and red absorbance maxima were evident, the signals being too weak to show any other minor absorbance peaks). There was insufficient material for isolation of these components to identify the esterifying alcohols by GC-MS after hydrolysis.

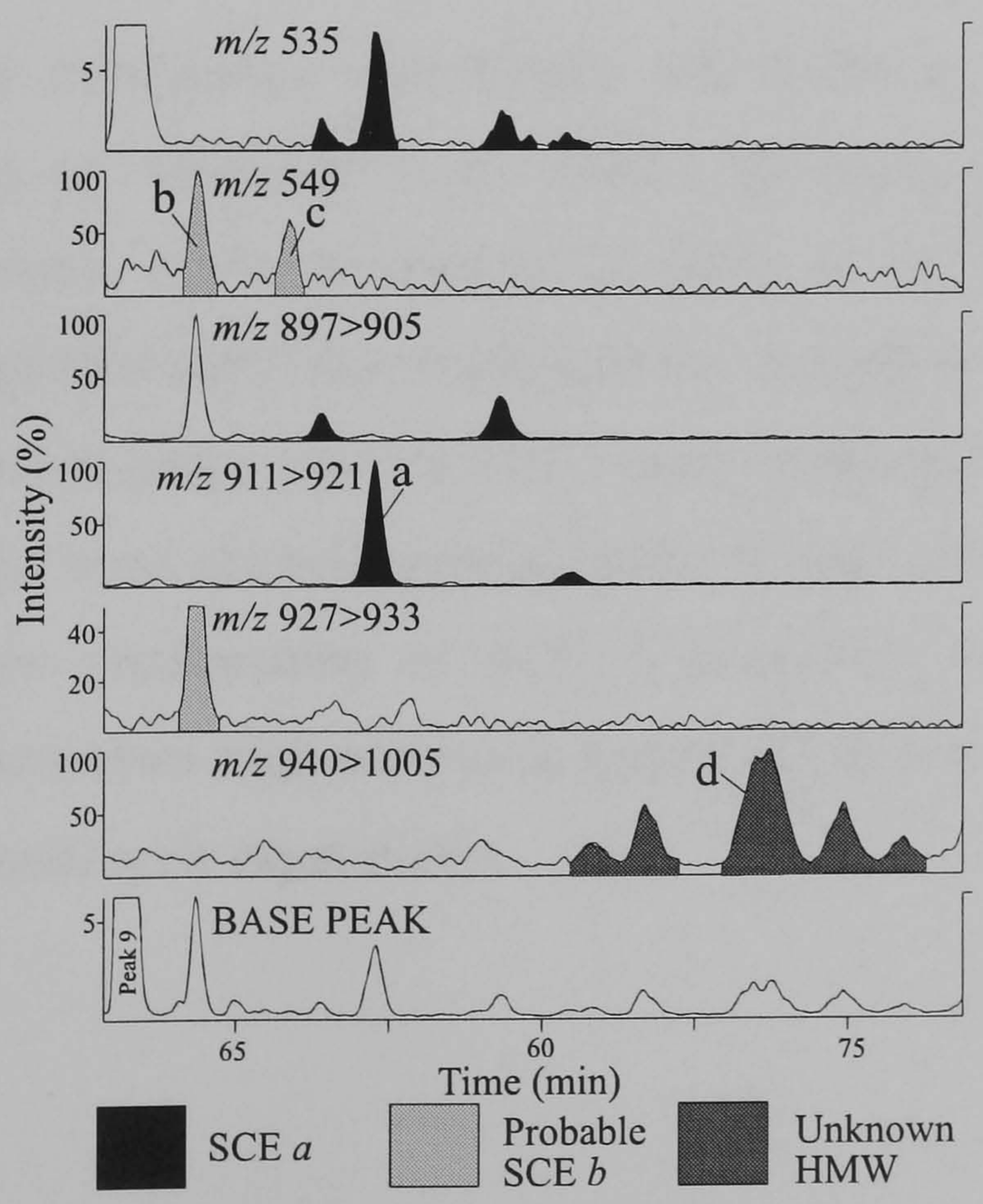


Figure 2-8. *Selected mass chromatograms from T. suecica faecal pellet SCE region.*

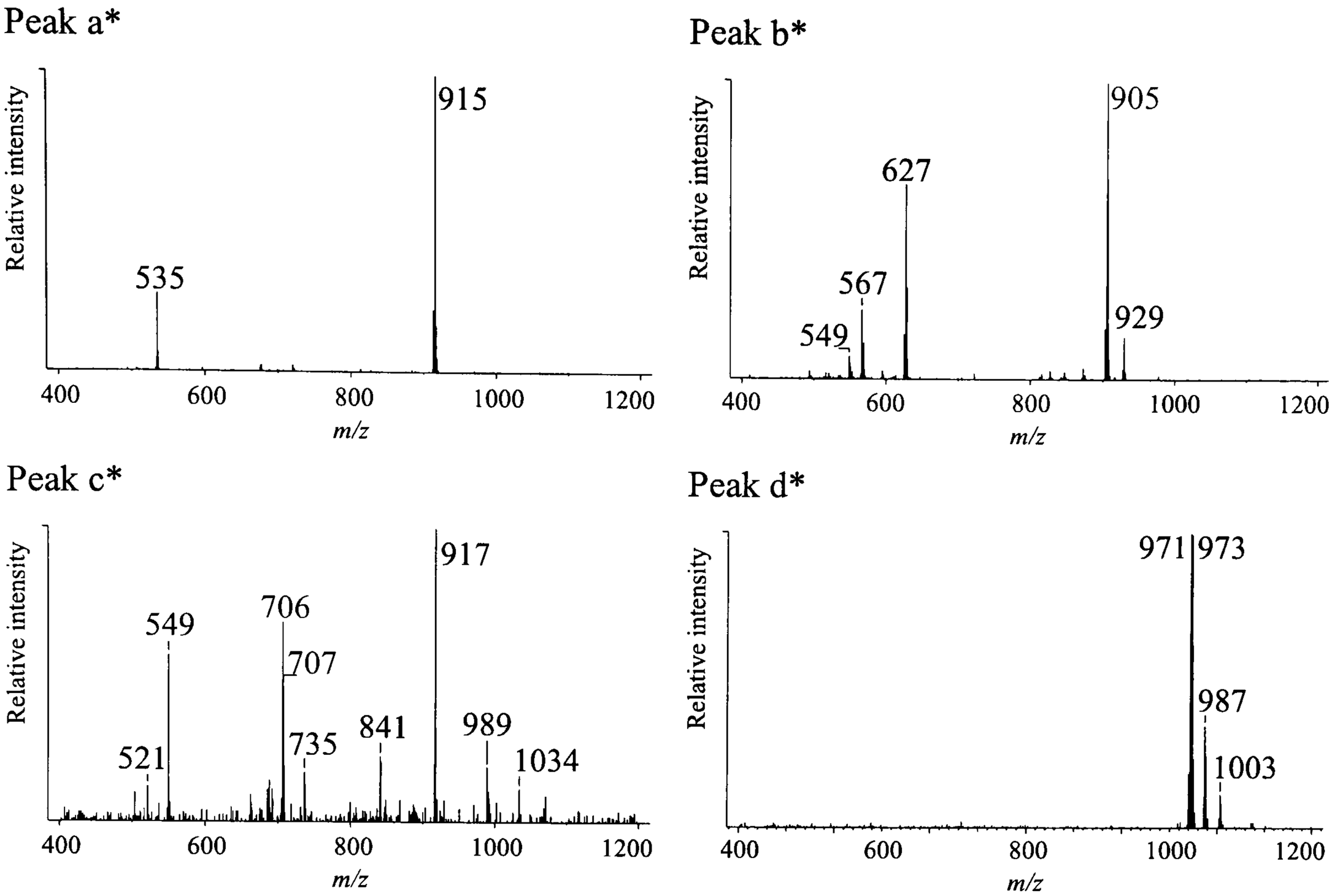


Figure 2-9. *Mass spectra of selected peaks in SCE region of T. suecica faecal pellets: (a) SCE a , MH^+= 915 - C_{28} sterol with 2 double bonds; (b) Possible SCE b, MH^+= 929; (c) Possible SCE b, MH^+= 917; (d) Unknown high molecular weight peak. (* see fig. 2-8.)*

2.2.3. Summary

The results of these experiments reconfirmed that SCEs *a* are produced during zooplankton herbivory (*cf.* Harradine *et al.*, 1996b). However, since the results were inconclusive with respect to the production of SCEs *b*, no further analyses were performed to investigate the sterol distributions of the animals or algae. It was decided that the alga most likely to give a positive SCE *b* result if repeated on a larger scale was *T. suecica*, based on the mass chromatography results. It was hoped that this experiment would not only allow confirmation of SCE *b* production but also might allow identification of the unknown high molecular weight components in the faecal pellet extracts of all of the small scale experiments.

2.4. STAGE 2: RESULTS AND DISCUSSION

2.4.1. Algal Culture

The HPLC chromatogram of the extract (400+430 nm; fig. 2-10) shows that chl *a* (peak 4, **I**) is present in significant abundance along with its C-13^2 epimer (4').

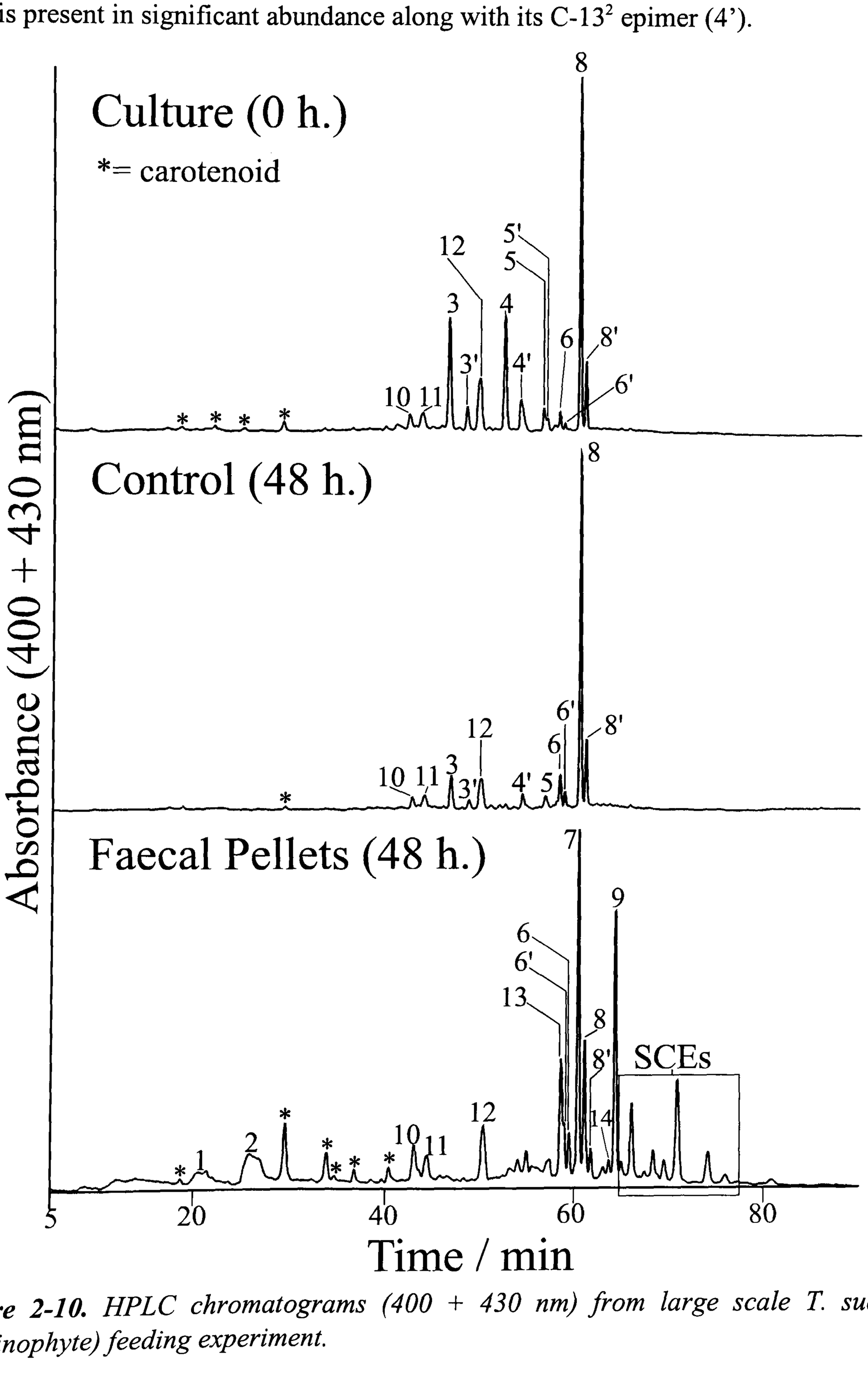


Figure 2-10. *HPLC chromatograms (400 + 430 nm) from large scale T. suecica (prasinophyte) feeding experiment.*

The mass spectrum of chl *a* (fig. 2-11) shows some differences from that in the small scale experiments due to the lower vaporiser temperature (450°C *vs.* 500°C), leading to full retention of the magnesium ion during fragmentation. The most significant differences (*cf.* fig. 2-5a) are the absence of the *m/z* 871, 593 and 533 ions at 450°C. In this case fragmentation gives ions at *m/z* 835 (loss of the C-13^2 carbomethoxy group from MH^+ at *m/z* 893), *m/z* 615 (loss of $C_{20}H_{38}$ from *m/z* 893), *m/z* 583 and 555 (loss of methanol or the C-13^2 carbomethoxy group, respectively, from *m/z* 615). The ion at *m/z* 673 is unassigned (but see below). The electronic spectrum ($\lambda_{max.}$ 380, 413, 431, 581, 617 and 662 nm) is similar to that given by Jeffrey *et al.* (1997) for a standard chl *a* (λ_{max} 383, 411, 430, 534, 580, 617 and 662 nm in acetone).

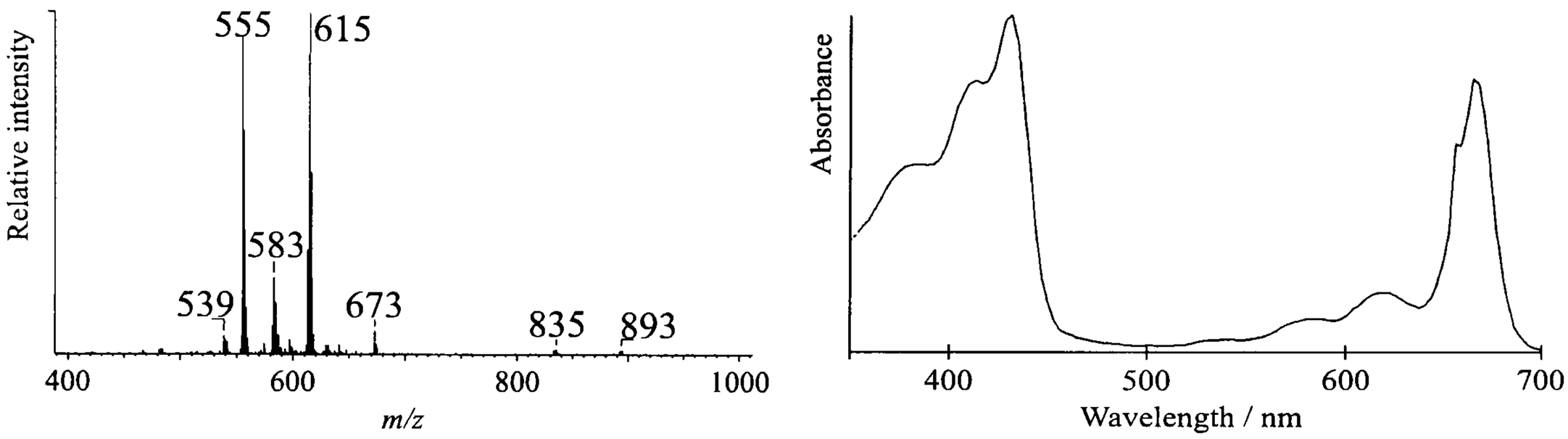


Figure 2-11. *Mass and electronic spectra of peak 4 (chl a)*

The mass spectrum of the epimer of chl *a* (fig. 2-12) is similar except for the absence of MH^+ presumably due to the lower relative abundance of the epimer. The ions at *m/z* 667 and 665 are unassigned and may be due to co-elution of an unidentified compound as they are not present in the spectrum of chl *a*. The electronic spectrum is identical to that of the major epimer (fig. 2-12).

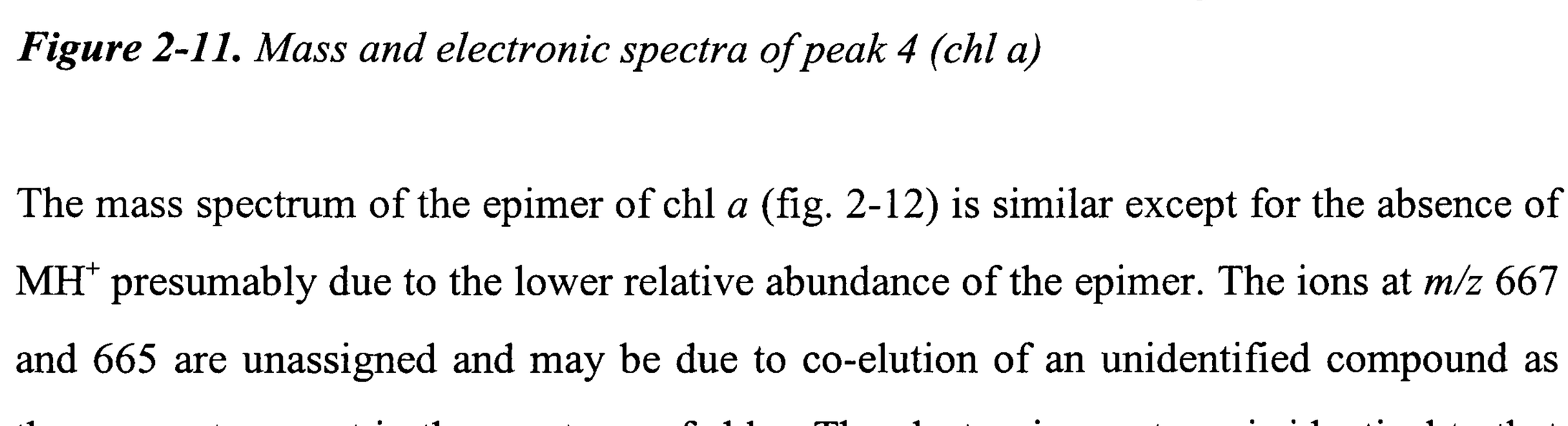


Figure 2-12. *Mass and electronic spectra of peak 4' (chl a')*

Peak 12 eluting prior to chl *a*, has an electronic spectrum similar to that of chl *a* ($\lambda_{max.}$ 419, 428, 530, 575, 614 and 662 nm; fig. 2-13). Mass chromatography revealed that the peak consisted of two co-eluting compounds with the ions *m/z* 587 and 543 eluting just prior to the ions at *m/z* 631, 613 and 571. The position at which these components elute i.e. prior to chl *a* suggested more polar species than chl *a*, possibly allomers. Although there is no indication of MH^+ at *m/z* 909, the ions at *m/z* 631 (loss of $C_{20}H_{38}$) with subsequent losses of Mg (with the addition of 2H) or C-13^2 carbomethoxy to give *m/z* 613 and 571, suggesting a chl *a* mono-oxygenated allomer; hence the later eluting component is assigned as C-13^2 OH chl *a* (**XII**). The ions originating from the earlier eluting component are assigned as fragments of C-15^1 hydroxylactone chl *a* (**XXX**). The MH^+ at *m/z* 925 is not present but *m/z* 587 is assigned as MH^+ - ($C_{20}H_{38}$ + CO_2Me) and *m/z* 543 as loss of CO_2 from *m/z* 587. The disruption of ring E in this lactone ring system leads to a hypsochromic shift in the Soret band (Soret max. 416 nm; Woolley *et al.*, 1998) and therefore accounts for the unusual structure of the electronic spectrum of this peak which shows a lack of structure on the blue side of the Soret band relative to that of chl *a* (*cf.* fig. 2-11).

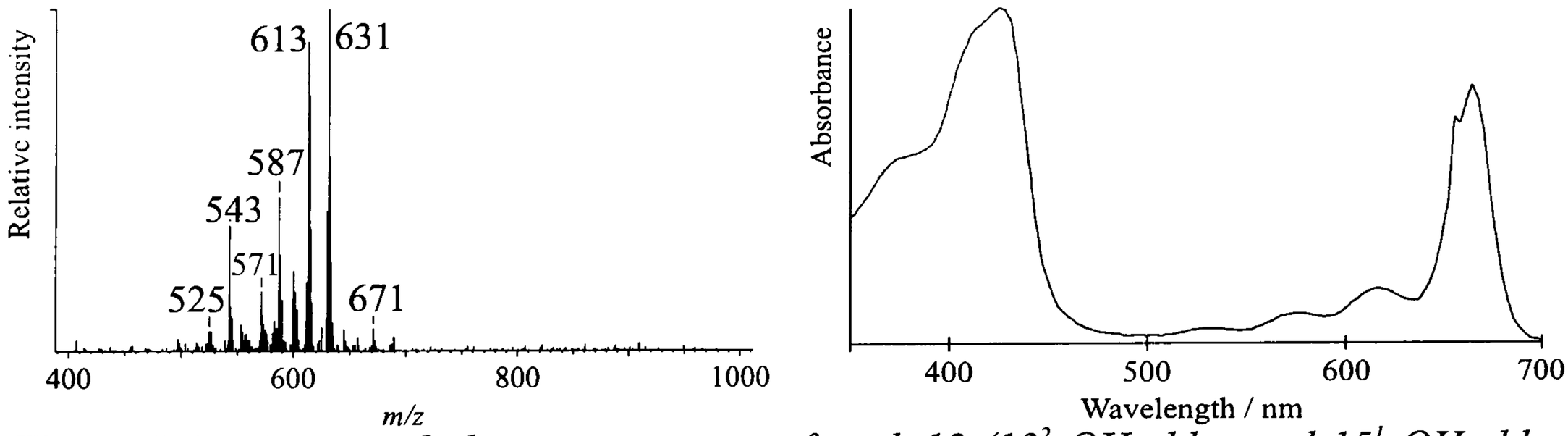


Figure 2-13. *Mass and electronic spectra of peak 12 (13^2-OH chl a and 15^1-OH chl a lactone).*

Also present in significant abundance are both C-13^2 epimers of chl *b* (peaks 3 and 3', **II**). The mass spectrum of chl *b* (fig. 2-14) is analogous to that for chl *a*, with MH^+ at *m/z* 907 again showing full retention of magnesium and fragmentation to give ions of *m/z* 629, 597 and 571. The ion at *m/z* 687 is analagous to the *m/z* 673 in the chl *a* spectrum, allowing for the extra oxygen, so it would seem that they are both genuine fragments. The electronic spectrum ($\lambda_{max.}$ 458, 596 and 647 nm) is similar to that given by Jeffrey *et al.* (1997) for a standard ($\lambda_{max.}$ 457, 597 and 646 nm in acetone).

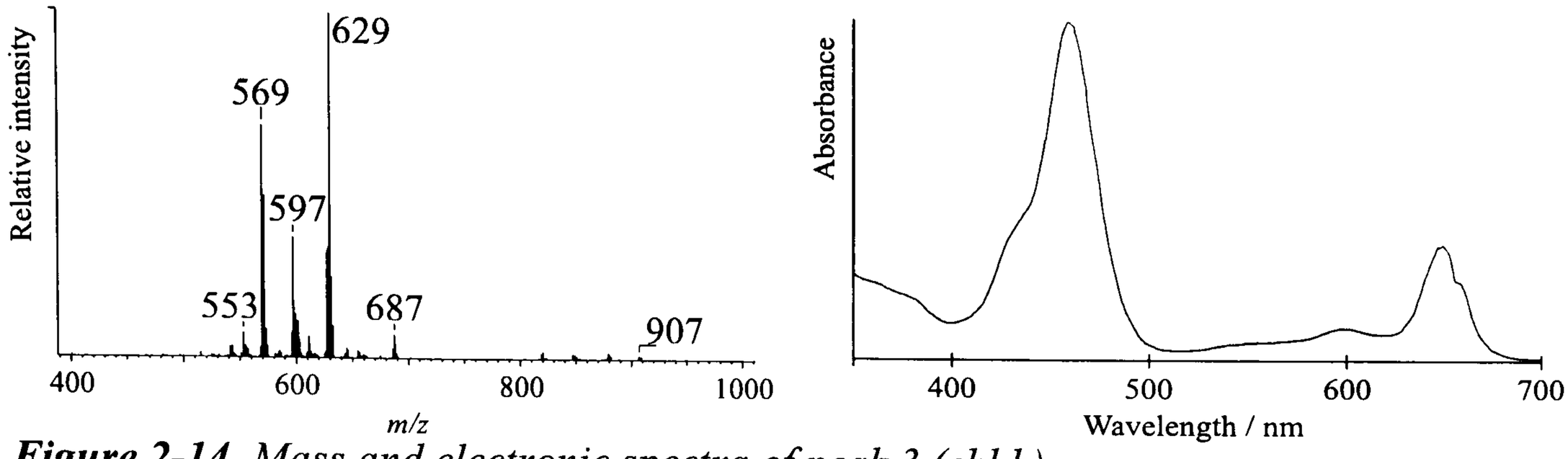


Figure 2-14. *Mass and electronic spectra of peak 3 (chl b).*

The mass spectrum of the epimer (peak 3'; fig 2-15) is similar except for a higher abundance of an ion at *m/z* 571 relative to *m/z* 569 again showing that the C-13^2 carbomethoxy group can be lost by two different mechanisms (see section 2.3.1.).

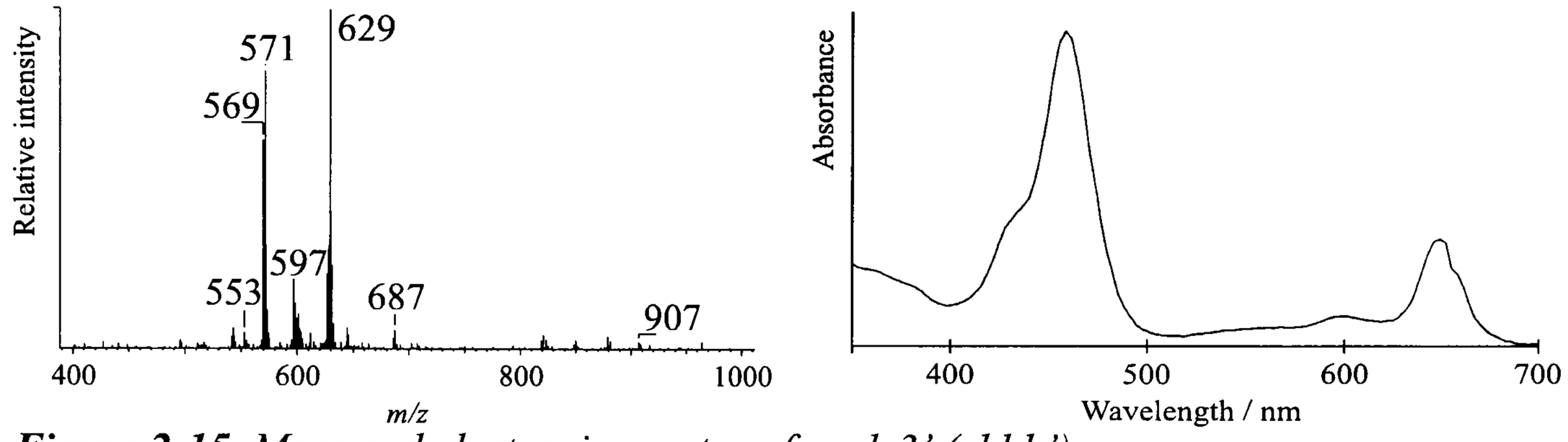


Figure 2-15. *Mass and electronic spectra of peak 3' (chl b').*

Peak 10 is related to chlorophyll b with the electronic spectrum ($\lambda_{max.}$ 449, 587 and 632 nm, fig. 2-16) showing a significant hypsochromic shift, thus suggesting a chl *b* lactone type structure (see above). The MH^+ at *m/z* 939 is not observed but the base peak at *m/z* 601 is assigned as $MH^+ - (C_{20}H_{38} + CO_2Me)$ and *m/z* 557 as loss of CO_2 from *m/z* 601 indicating peak 10 is C-15^1 hydroxylactone chl *b* (**XXXI**). A number of other ions were present indicating an unknown co-eluting component.

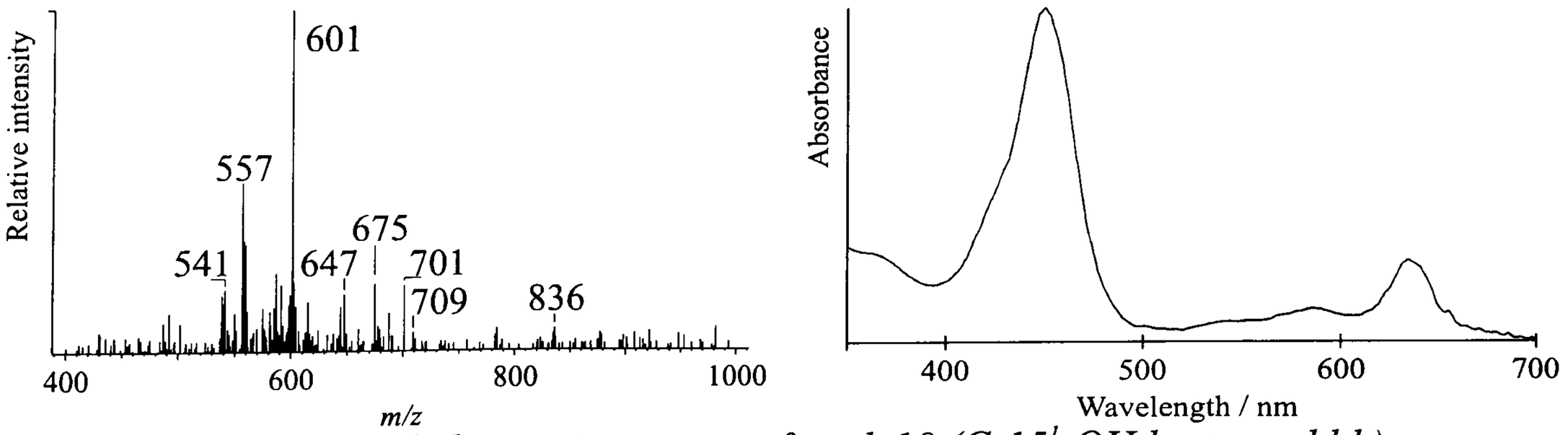


Figure 2-16. *Mass and electronic spectra of peak 10 (C-15^1 OH lactone chl b).*

The electronic spectrum (fig. 2-17) of peak 11 is identical to that of chl *b*. The base peak at *m/z* 645 is assigned as loss of $C_{20}H_{38}$ from MH^+ (*m/z* 923; not observed) with *m/z* 627 and 585 (loss of H_2O and C-13^2 carbomethoxy from *m/z* 645 respectively) supporting the assignment of peak 11 as C-13^2 hydroxychl *b* (**XXXII**).

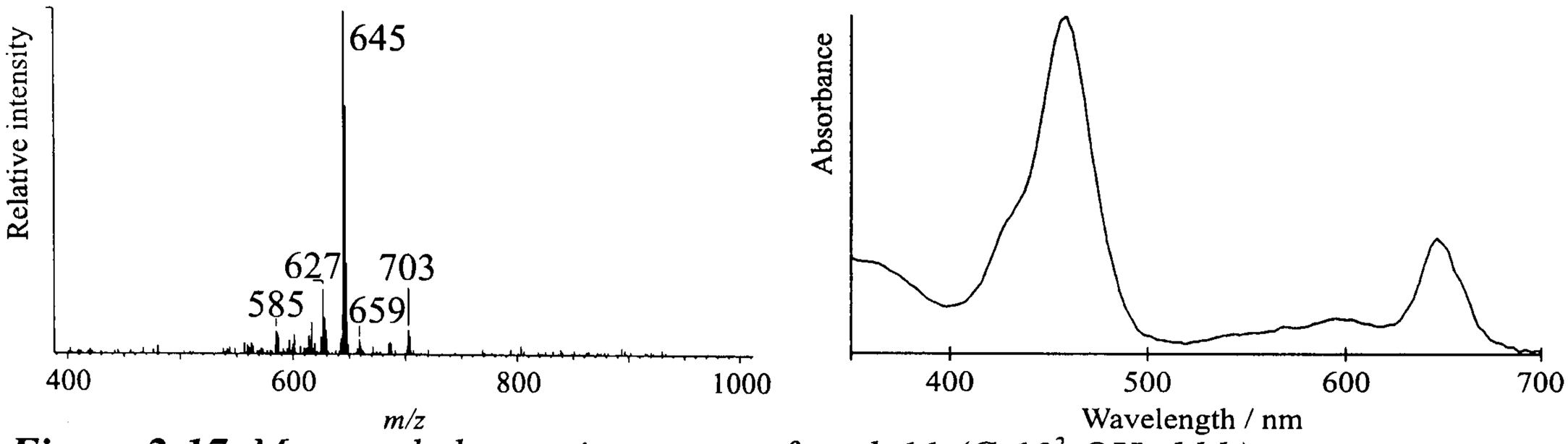


Figure 2-17. *Mass and electronic spectra of peak 11 (C-13^2 OH chl b).*

The most abundant chl *a* derivative is phaeophytin *a* (8, **VIII**) and its C-13^2 epimer (8'). The mass spectrum (fig. 2-18) shows an abundant MH^+ at *m/z* 871 with the expected fragments at *m/z* 839, 813, 593 due to loss of methanol, C-13^2 carbomethoxy and $C_{20}H_{38}$ respectively, and the ions at *m/z* 561 and 533 due to loss of methanol and C-13^2 carbomethoxy from *m/z* 593; *m/z* 517 is tentatively assigned as loss of OH from *m/z* 533 with the addition of a hydrogen. The electronic spectrum ($\lambda_{max.}$ 407, 506, 533, 606 and 665 nm; fig 2-18) is similar to that of a standard (*cf.* Jeffrey *et al.*, 1997; $\lambda_{max.}$ 410, 505, 535, 609 and 666 nm in acetone). The mass and electronic spectra of the epimer (peak 8'; fig. 2-19) are similar to those of the major isomer.

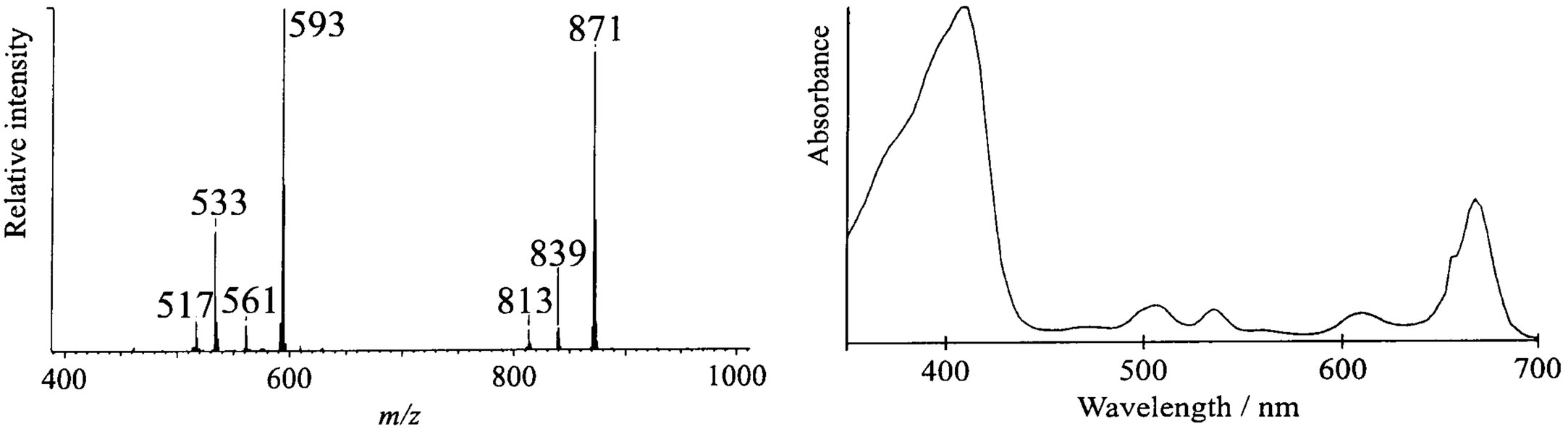


Figure 2-18. *Mass and electronic spectra of peak 8 (phaeophytin a).*

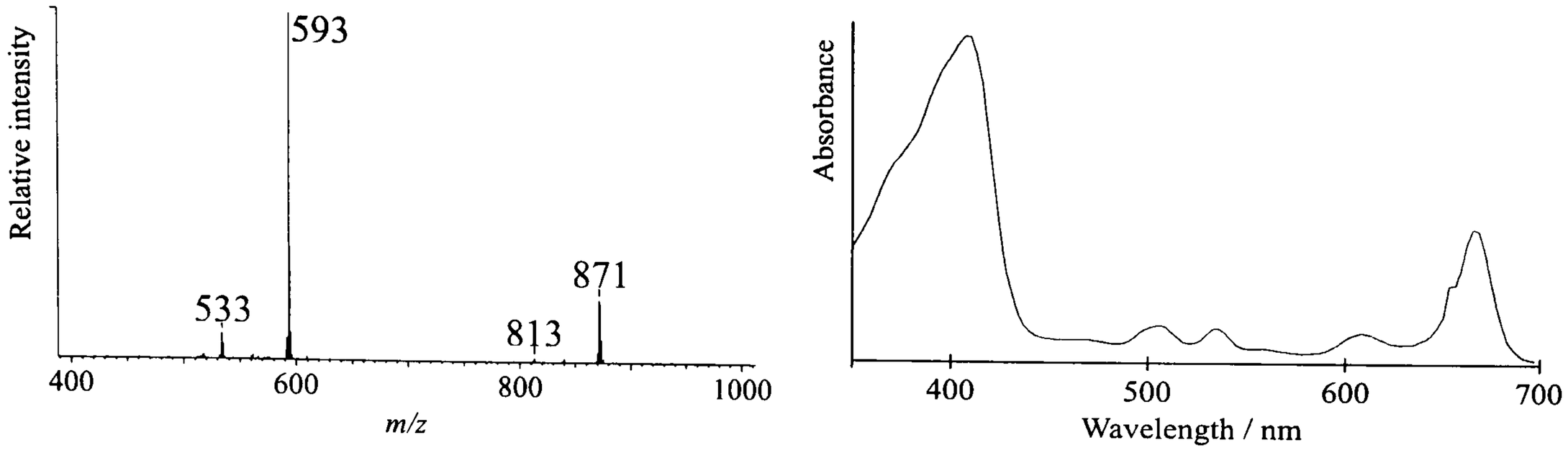


Figure 2-19. *Mass and electronic spectra of peak 8' (phaeophytin a').*

The spectrum (fig. 2-20) of the minor peak 6 has MH^+ at *m/z* 887, 16 mass units higher than of phaeophytin *a*, and is consistent with C-13^2 hydroxyphaeophytin *a* (**XIII**), with fragment ions at *m/z* 609 (loss of $C_{20}H_{38}$), and *m/z* 869 and 591 (loss of H_2O from *m/z* 887 and 609 respectively) and *m/z* 559 and 531 (loss of MeOH or C-13^2 carbomethoxy from *m/z* 591, respectively) matching those observed previously (Harris *et al.*, 1995a). The electronic spectrum is similar to that of phaeophytin *a*.

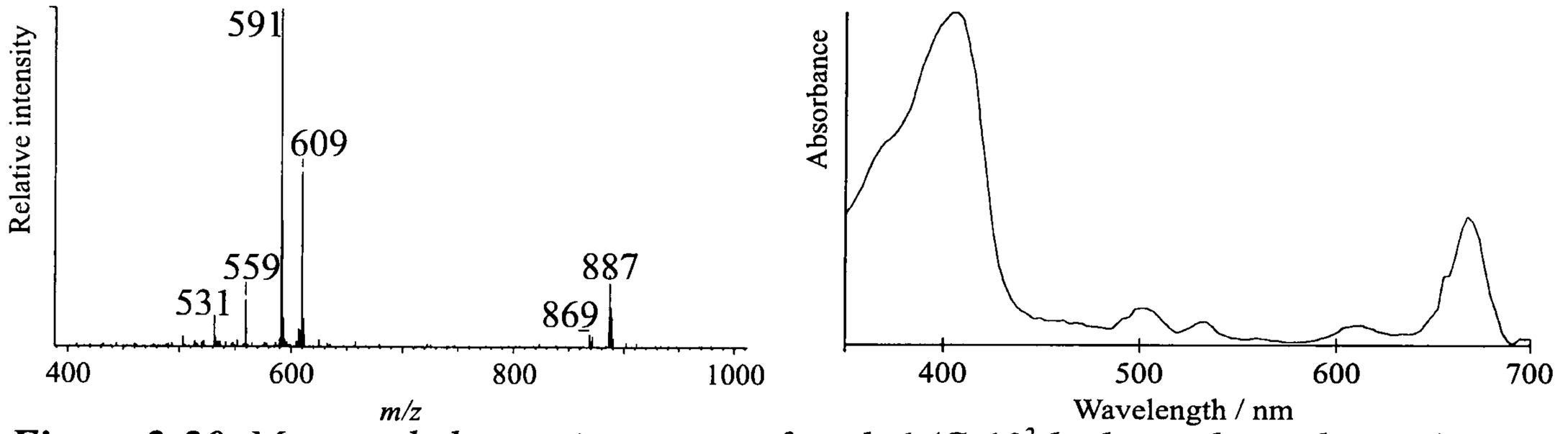


Figure 2-20. *Mass and electronic spectra of peak 6 (C-13^2 hydroxyphaeophytin a).*

Both the mass and electronic spectra of the C-13^2 epimer (peak 6') are similar to those of the major isomer with the exception of an ion at *m/z* 629 which could not be assigned (fig. 2-21).

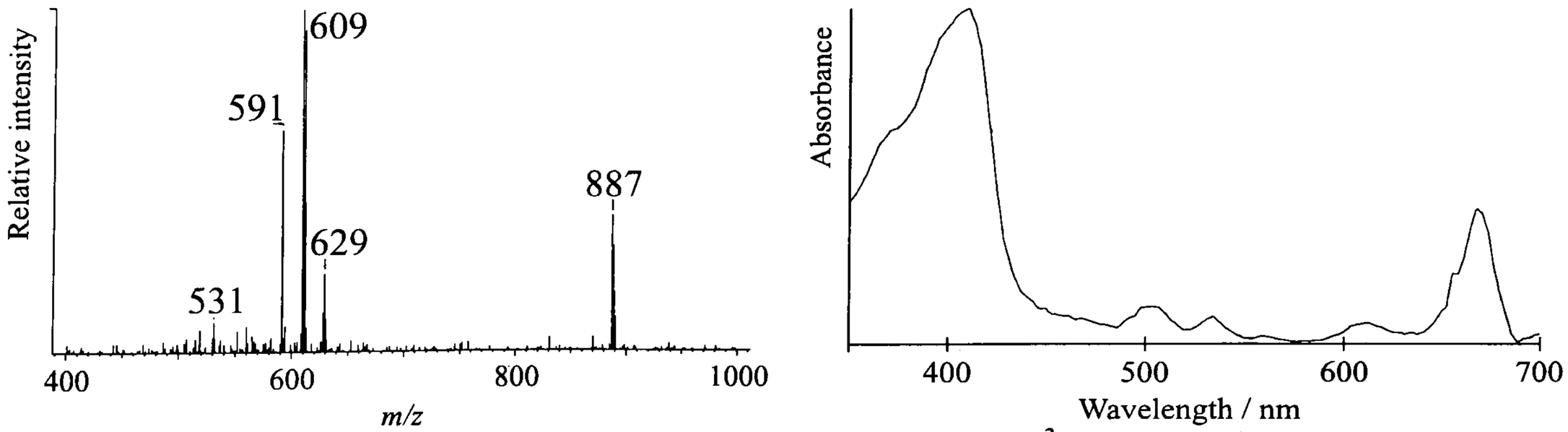


Figure 2-21. *Mass and electronic spectra of peak 6' (C-13^2 hydroxyphaeophytin a').*

A minor peak tentatively identified as containing phaeophytin *b* (5, **XXVIII**) as well as a significant unknown component was observed along with the tentatively identified C-13^2 epimer (5') of phaeophytin *b*. The mass spectrum (fig. 2-22) shows a number of significant ions which could not be assigned, however, a possible MH^+ at *m/z* 885 with fragment ions of *m/z* 607 (loss of $C_{20}H_{38}$) and *m/z* 589 (loss of H_2O from *m/z* 607) support the phaeophytin *b* assignment. The electronic spectrum ($\lambda_{max.}$ 434 and 668 nm) shows only a weak relationship to that given for phaeophytin *b* by Jeffrey *et al.* (1997; $\lambda_{max.}$ 370, 434, 528, 600 and 653 nm in acetone), presumably due to the co-eluting component and the methanol and water as well as acetone in the HPLC eluent.

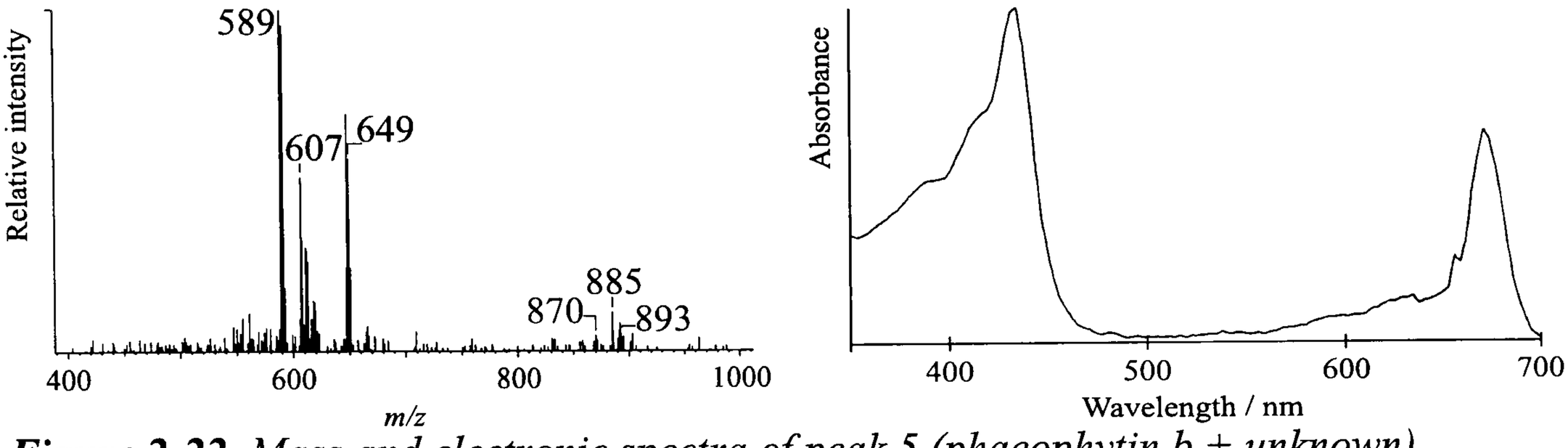


Figure 2-22. *Mass and electronic spectra of peak 5 (phaeophytin b + unknown).*

A number of other minor chlorins were observed along with several carotenoids outwith the scope of this study. The absence of chlorophyllide *a* or *b* in the extract is consistent with the known lack of the enzyme chlorophyllase in this organism (Jeffrey and Hallegraeff, 1987). The chlorin data for this large scale experiment is summarised in Table 2-1.

2.4.2. Algal Control.

The chromatogram of the control extract (fig. 2-10) is essentially similar to that of the fresh culture; however, the relative abundance of the chls *a* and *b* is significantly reduced, with chl *a* (peak 4) being absent and phaeophytin *a* (8 and 8') being by far the most abundant component. There is a slight increase in the abundance of the mono-oxygenated allomer (peak 6) relative to phaeophytin *a* (8 and 8'). The four allomers of chls *a* (peak 12) and *b* (10 and 11) were also present along with a reduced number of carotenoids relative to the culture.

Peak*	MH^+	Other ions	λ_{max} /nm	Assignment	Structure
1	549		437,524,599,653	pyrophaeophorbide *b*	**XX**
2	535		410,506,536,608,665	pyrophaeophorbide *a*	**X**
3	907	687,629,597,571, 569,533	458,596,647	chlorophyll *b*	**II**
3'	907	687,629,597,571, 569,533	458,599,647	chlorophyll *b* epimer	**II**
4	893	665,615,583,555	431,581,617,66,	chlorophyll *a*	**I**
4'	893	665,615,583,555	431,587,620,665	chlorophyll *a* epimer	**I**
5	885	649,607,591	434,668	*phaeophytin b* + *unknown*	**XXVIII**
5'	*885*		434,671	*phaeophytin b epimer*	**XXVIII**
6	887	641,623,609,591, 559	404,500,530,611,665	hydroxyphaeophytin *a* + unknown	**XIII**
6'	887	628,610,591	410,503,533,611,665	hydroxyphaeophytin *a* epimer	**XIII**
7	827	799,549,521,	434,530,599,656	pyrophaeophytin *b*	**XXIX**
8	871	839,813,593,561, 533,517	407,506,533,602,665	phaeophytin *a*	**VIII**
8'	871	839,813,593,561, 533,517	410,506,533,602, 665	phaeophytin *a* epimer	**VIII**
9	813	535	410,470,506,536, 608,665	pyrophaeophytin *a*	**XI**
10	(939)	836,709,675,647, 601,557,541		C-15[1] OH lactone chlorophyll *b*	**XXXI**
11	(923)	703,659,645,627, 585		C-13[2] OH chlorophyll *b*	**XXXII**
12[1]	(925 and 909)	671,631,613,603, 587,571,534,525	(419),428,530, 575,614,662	C-15[1] OH lactone chlorophyll *a* and C-13[2] OH chlorophyll *a*	**XXX** **XII**
12[2]		909,689,631,613, 587,571,543,523	419,569,611,656	C-15[1] OH lactone chlorophyll *a* and C-13[2] OH chlorophyll *a*	**XXX** **XII**
13	917 and 903	887,639,625,609, 565	398,497,527,611,668	C-15[1] OH lactone phaeophytin *a* and *C-13[2] OMe phaeophytin a*	**XXXIII** **XXXIV**
14	(843)	565	407,542,584,644,695	purpurin-18-phytyl ester	**XIVa**

* see fig. 2-10; [1] in culture and control; [2] in pellets; brackets indicate MH^+ not observed; assignments in italics tentative.

Table 2-1. *Chlorin data from large scale T. suecica experiment.*

2.4.3. Faecal Pellets

The faecal pellet extract after 48 h feeding (fig. 2-10) shows a significantly more complex distribution of pigments than either the culture or algal control. Of the components not in the culture or control the most abundant chl *a* related component is pyrophaeophytin *a* (peak 9, **XI**). This has a simple mass spectrum (fig. 2-23) consisting of MH^+ at *m/z* 813 and a single fragment ion of *m/z* 535 (loss of $C_{20}H_{38}$). The electronic spectrum ($\lambda_{max.}$ 410, 470, 506, 536, 608 and 665 nm) is similar to that of a standard (*cf.* Jeffrey *et al.*, 1997, $\lambda_{max.}$ 410, 507, 536, 609 and 667 nm in acetone).

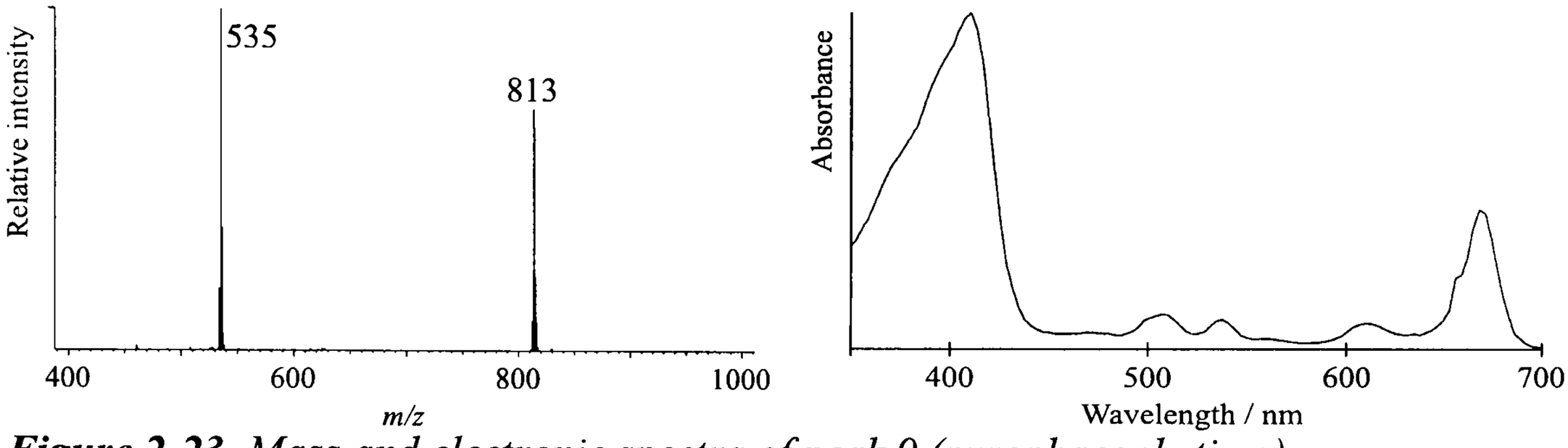


Figure 2-23. *Mass and electronic spectra of peak 9 (pyrophaeophytin a).*

The mass spectrum of the minor, poorly resolved peak (2, **X**), consists of a single ion at *m/z* 535, indicating pyrophaeophorbide *a* (fig. 2-24). This component is poorly resolved due to the presence of a free acid group, which results in peak broadening under the reversed phase HPLC conditions used. Previously (e.g. Harradine *et al.*, 1996b) this problem has been overcome by the use of diazomethane for derivatisation prior to analysis, but this method was abandoned in the present study due to the high occurrence of oxygenated artifacts in methylated extracts (see Chapter 4). The electronic spectrum, although of low intensity, is similar to that of a standard ($\lambda_{max.}$ 410, 506, 536, 608 and 665 nm).

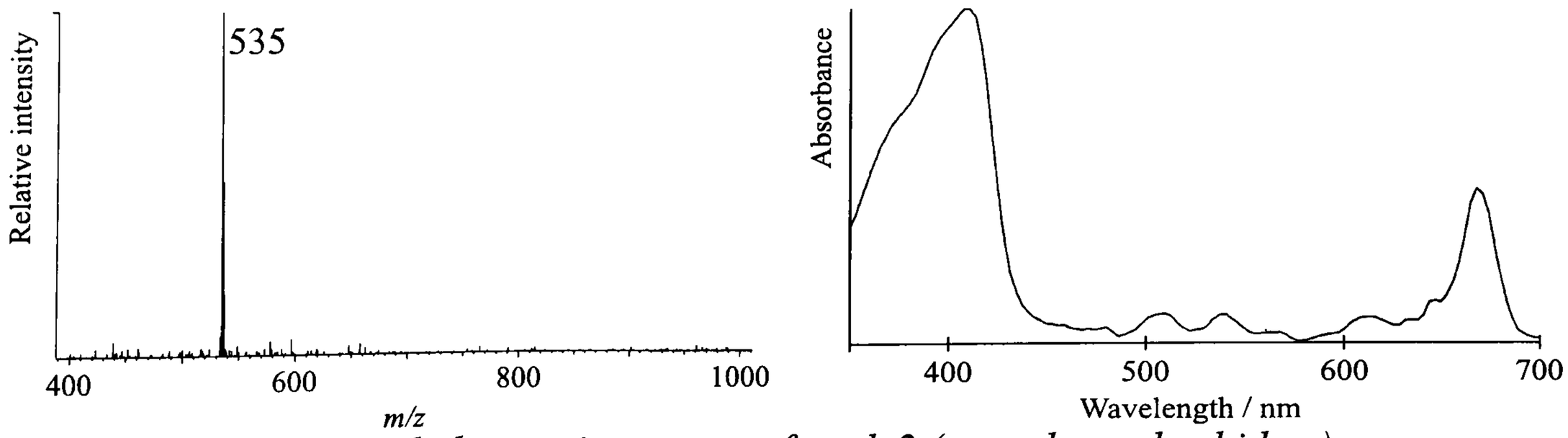


Figure 2-24. *Mass and electronic spectra of peak 2 (pyrophaeophorbide a).*

The major chl *b* alteration products include pyrophaeophytin *b* (peak 7, **XXIX**) not co-eluting with phaeophytin *a* due to a slight alteration of the HPLC solvent gradient to allow the two species to be resolved (see chapter 8). The mass spectrum (fig. 2-25) consists simply of MH^+ at *m/z* 827, a major fragment ion at *m/z* 549 (loss of $C_{20}H_{38}$) and the minor ions at *m/z* 799 and 521, tentatively assigned as loss of the C-7 formyl group $[M\text{-}CHO+H]^+$. The electronic spectrum ($\lambda_{max.}$ 434, 530, 599 and 656 nm) is similar to that given by Jeffrey *et al.* (1997) for phaeophytin *b* (see above).

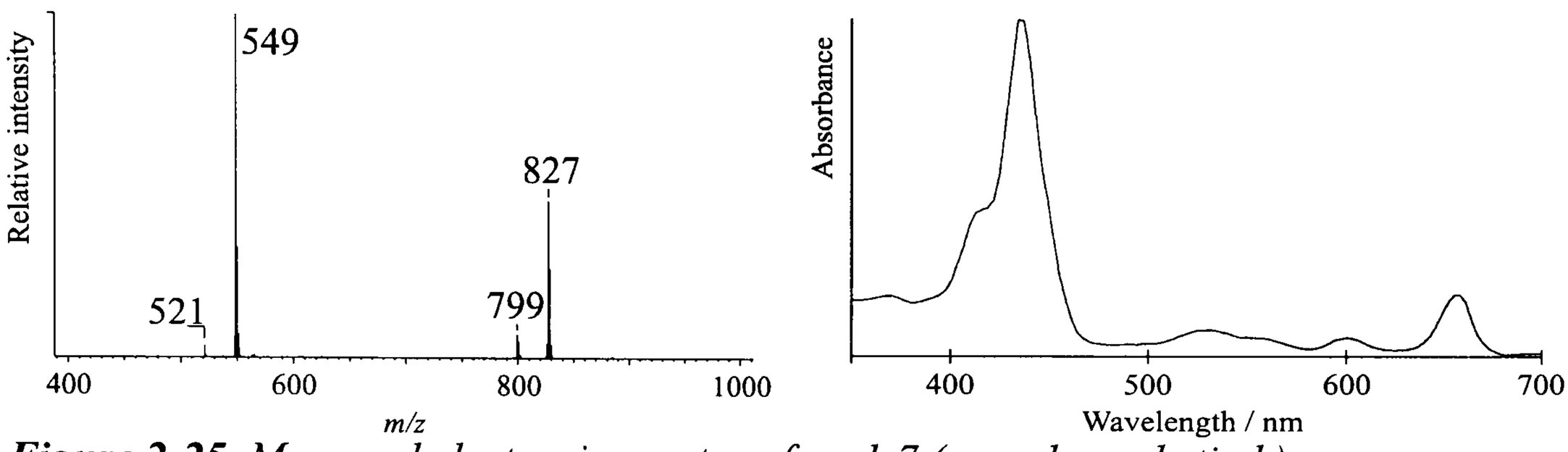


Figure 2-25. *Mass and electronic spectra of peak 7 (pyrophaeophytin b).*

The mass spectrum of a minor, poorly resolved peak (1; fig. 2-26), was found to consist simply of a base peak at *m/z* 549 indicative of pyrophaeophorbide *b* (**XX**) and with the ion at *m/z* 975 presumably resulting from co-elution from an unknown component. The electronic spectrum, although of low intensity, was similar to that for pyrophaeophytin *b* ($\lambda_{max.}$ 437, 524, 599 and 653 nm).

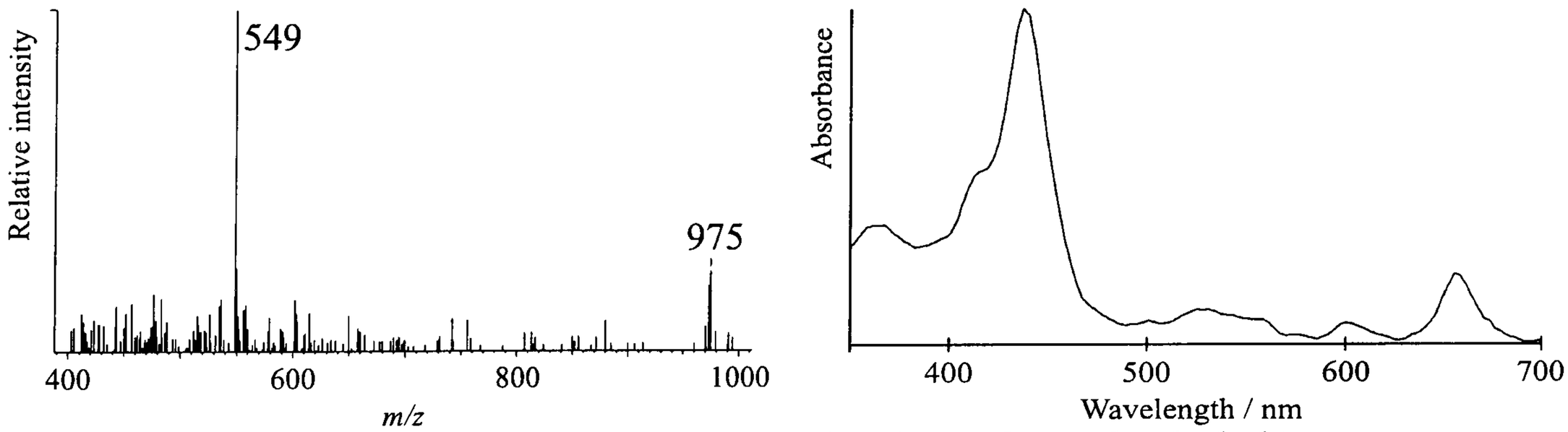


Figure 2-26. *Mass and electronic spectra peak 1 (pyrophaeophorbide b).*

There has clearly been some change in the relative abundances of the two co-eluting chl *a* allomers (12) evident in the relative abundance of the ions in the mass spectrum and the change in the structure of the electronic spectrum ($\lambda_{max.}$ 419, 575, 614 and 662 nm;

fig. 2-27) indicating a greater proportion of the lactone (**XXX**) relative to the mono-oxygenated allomer (**XII**).

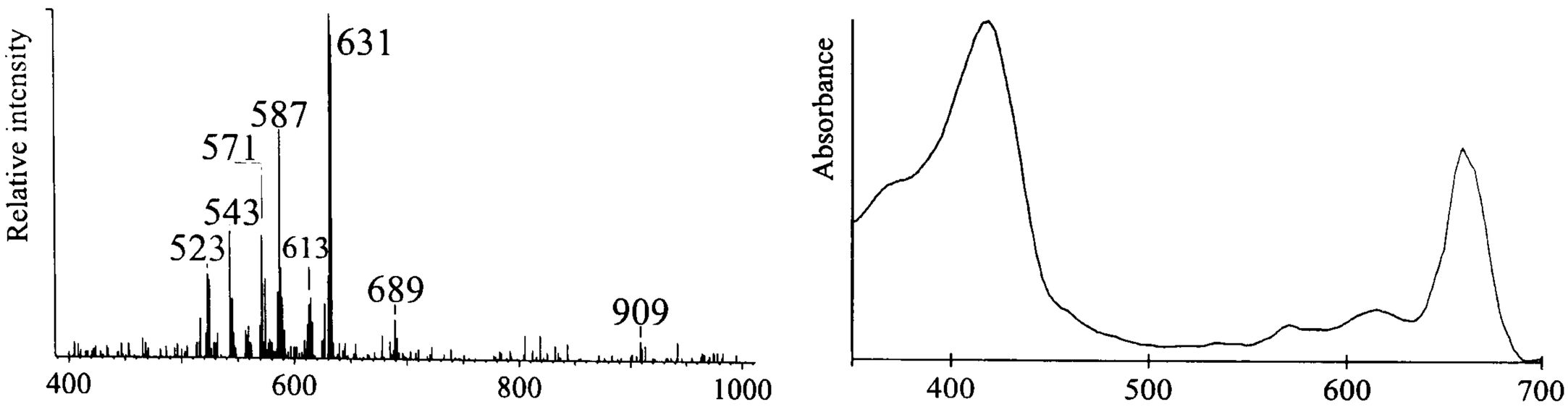


Figure 2-27. *Mass and electronic spectra of peak 12 (faecal pellet extract; C-15[1] OH lactone chl a and C-13[2] OH chl a).*

Another chlorin component was detected partially co-eluting with the earlier eluting epimer of C-13^2 hydroxyphaeophytin *a* (peak 6). The electronic spectrum of this peak (13; fig 2-28), although generally similar to that of the well known chl *a* transformation products, showed hypsochromic shifts of *ca.* 9 nm in the 3 shorter wavelength absorbance maxima ($\lambda_{max.}$ 398, 497, 527, 611 and 668 nm). The possible MH^+ ions at *m/z* 903 and 917 suggests the component could be a mixture of C-15^1 hydroxy- and methoxylactones of phaeophytin *a*; the hypsochromic shift being consistent with that observed for the chl *a* and *b* lactones (see above). The ion at *m/z* 887 (loss of OMe+H from *m/z* 917) presumably could also originate from the co-eluting C-13^2 hydroxyphaeophytin *a* (peak 6). The ions at *m/z* 639 and 625 result from loss of $C_{20}H_{38}$ from *m/z* 917 and 903 respectively with subsequent loss of OMe(+H) from *m/z* 639 to give *m/z* 609 and loss of C-13^2 carbomethoxy from *m/z* 625 to give *m/z* 565. This suggests that the C-13^2 OMe group is lost more readily that the C-13^2 OH group. Therefore peak 13 is assigned as a mixture of C-15^1 hydroxy lactone phaeophytin *a* (**XXXIII**) and C-15^1 methoxy lactone phaeophytin *a* (**XXXIV**). The assignment of the C-15^1 methoxy lactone must be considered tentative, however, as its origin unclear as at no point during the extraction procedure or work-up prior to analysis was the sample exposed to a methanolic solution, usually considered to be the source of methylated allomers.

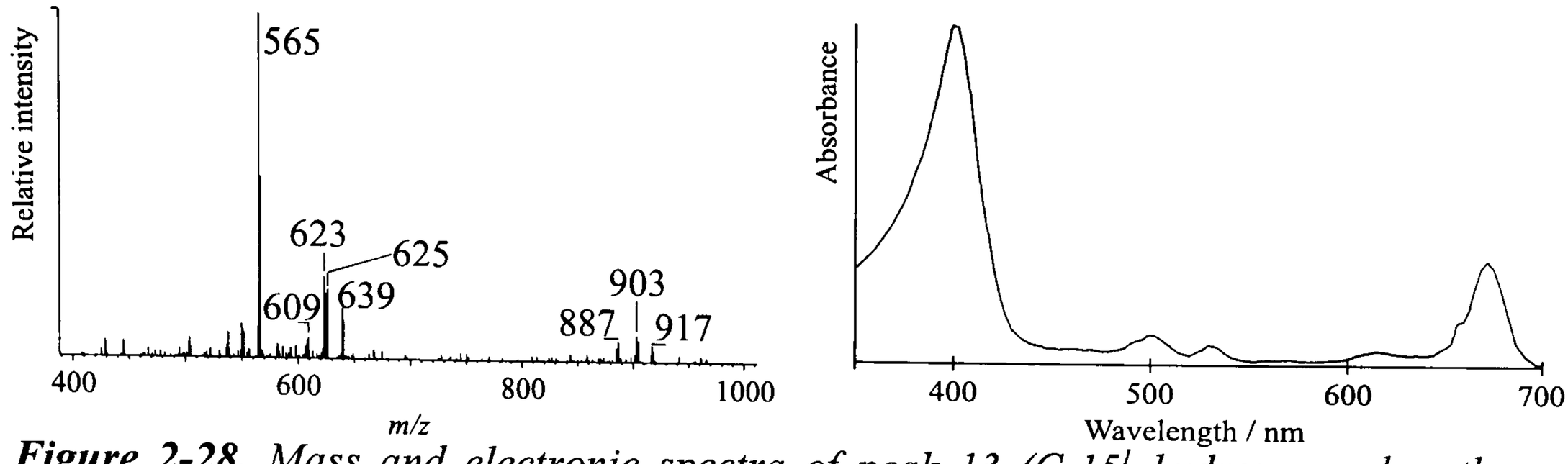


Figure 2-28. *Mass and electronic spectra of peak 13 (C-15[1] hydroxy- and methoxy-phaeophytin a lactone).*

The minor peak (14, **XIVa**) eluting just prior to pyrophaeophytin *a* gave mass and electronic spectra indicating a purpurin-like component (fig. 2-29). The only significant ion in the mass spectrum is *m/z* 565, assigned as the purpurin-18 moiety; however, based on the position at which this component elutes it is assigned as the purpurin-18-phytyl ester despite the absence of MH^+ of *m/z* 843 (*cf.* Naylor and Keely, 1998; see also Chapter 4). The electronic spectrum ($\lambda_{max.}$ 407, 542, 584, 944 and 695 nm) is highly diagnostic of the diketocyclic lactone exocycle specific to the purpurin-18 (**XIVb**) chromophore and is similar to that given by Louda *et al.* (1998; $\lambda_{max.}$ 408, 546 and 698).

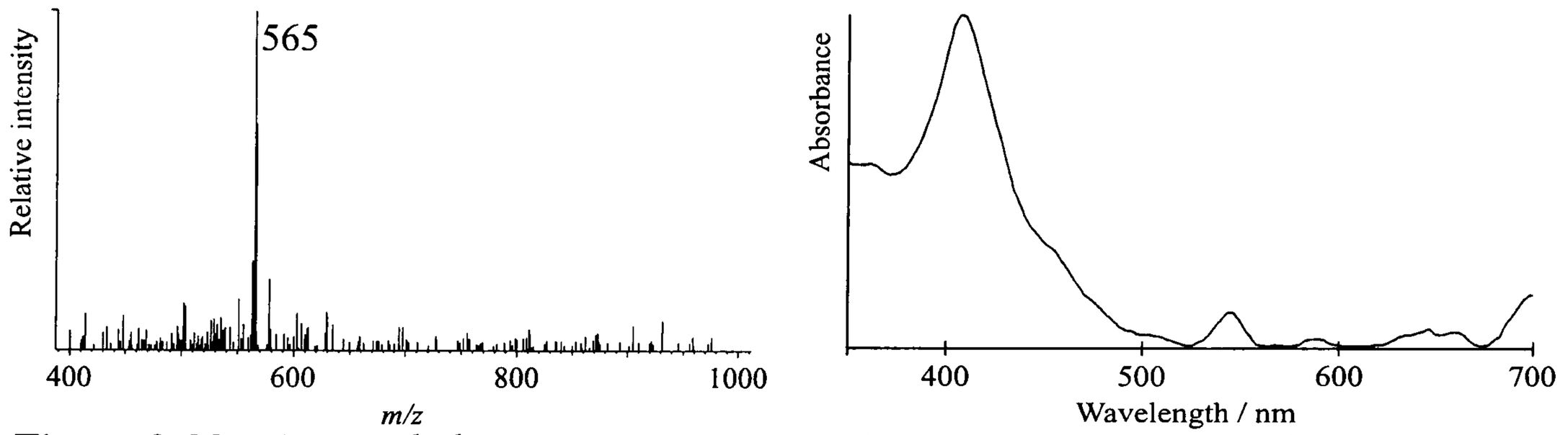


Figure 2-29. *Mass and electronic spectra of peak 15 (purpurin-18-phytyl ester).*

Eluting after pyrophaeophytin *a* is a group of peaks in the SCE region. Mass chromatography (fig. 2-30) revealed the presence of 5 peaks in the *m/z* 549 chromatogram, indicating the pyrophaeophorbide *b* moiety, and each corresponding to a peak in the 430 nm HPLC chromatogram, along with 5 in the *m/z* 535 chromatogram, indicating the pyrophaeophorbide *a* moiety, and each corresponding to a peak in the 400 nm HPLC chromatogram. The MH^+ ions of these peaks are also evident in the mass chromatograms (*m/z* 901, 903, 915, 917, 927 and 929), indicating the presence of 3 C_{27} sterols and 2 C_{28} sterols each esterified to pyrophaeophorbide *a* and pyrophaeophorbide

b (Table 2-2). The unshaded peaks in the *m/z* 903, 917 and 931 mass chromatograms originate from the isotope ions containing 2 ^{13}C atoms.

Pyrophaeophorbide *a* SCE MH^+	**Pyrophaeophorbide *b* SCE $M+H^+$**	**Esterifying sterol**
901(a)	915(a)	C_{27} 2 d.b.
901(b)	915(b)	C_{27} 2 d.b.
903	917	C_{27} 1 d.b.
915	929	C_{28} 2 d.b.
917	931	C_{28} 1 d.b.

Table 2-2. MH^+ *ions of pyrophaeophorbide a and b esters and* C_n *and degree of unsaturation of esterifying sterol.*

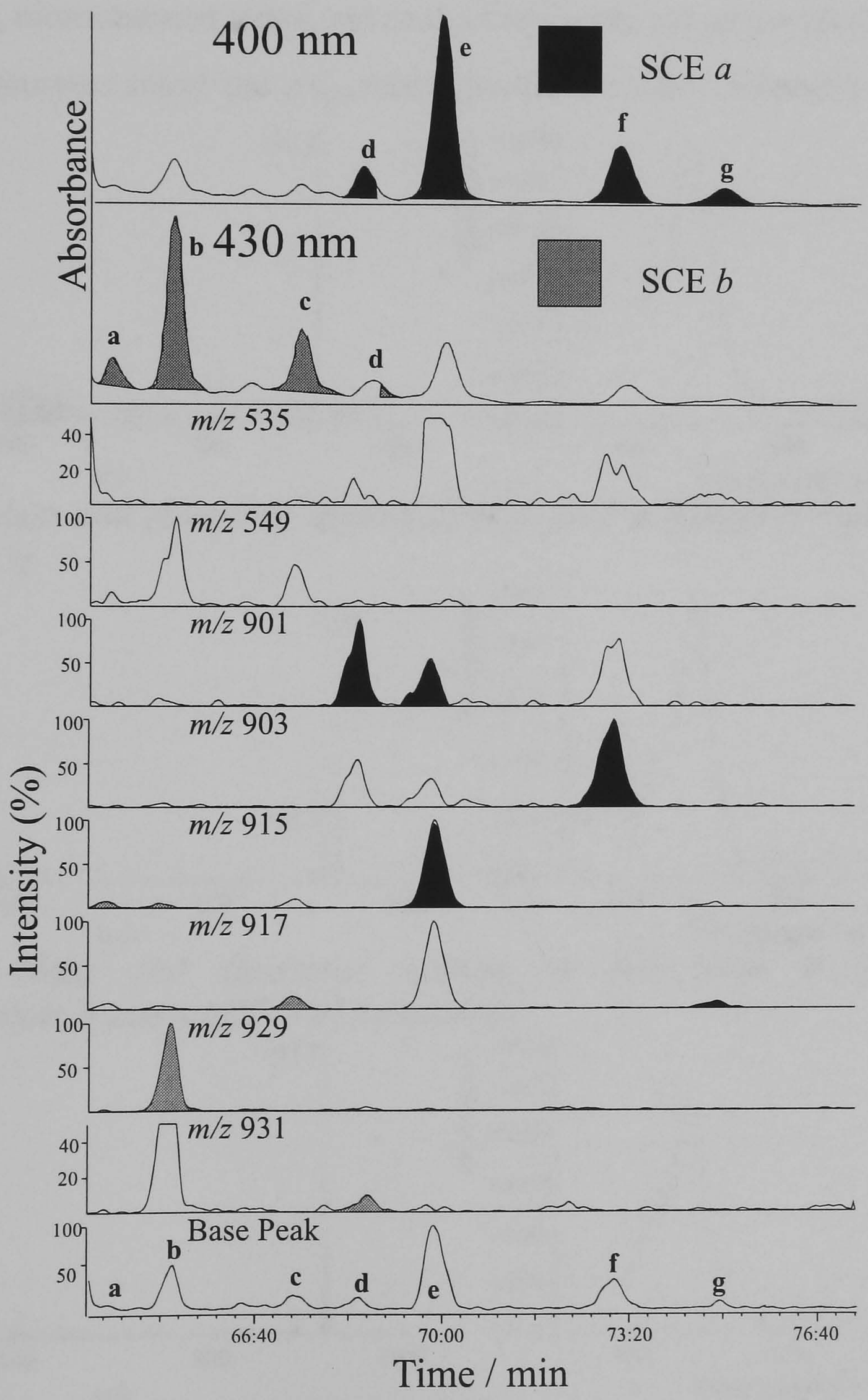


Figure 2-30. *HPLC chromatograms (400 nm and 430 nm) and mass chromatograms of faecal pellet SCE region.*

The mass and electronic spectra confirm the presence of 5 SCEs *a* and 5 SCEs *b* (figs 2-31 to 2-37). Peak a (fig. 2-30) consists of a single pyrophaeophorbide *b* ester esterified to a C_{27} diunsaturated sterol. Peak b comprises two co-eluting pyrophaeophorbide *b* esters of a second C_{27} diunsaturated sterol and a C_{28} diunsaturated sterol. The third SCE *b* peak (c) shows a single MH^+ indicating a C_{27} monounsaturated sterol. Peak d shows ions at both *m/z* 535 and 549 indicating co-eluting SCEs *a* and *b*. The MH^+ ions *m/z* 931 and 901 are assigned as a pyrophaeophorbide *b* ester of a C_{28} monounsaturated sterol and a pyrophaeophorbide *a* ester of a C_{27} diunsaturated sterol (see section 2.4.4.). Peak e consists of two co-eluting pyrophaeophorbide *a* esters of a second C_{27} diunsaturated sterol and a C_{28} diunsaturated sterol and peaks f and g are pyrophaeophorbide *a* esters of a C_{27} monounsaturated sterol and a C_{28} monounsaturated sterol, respectively.

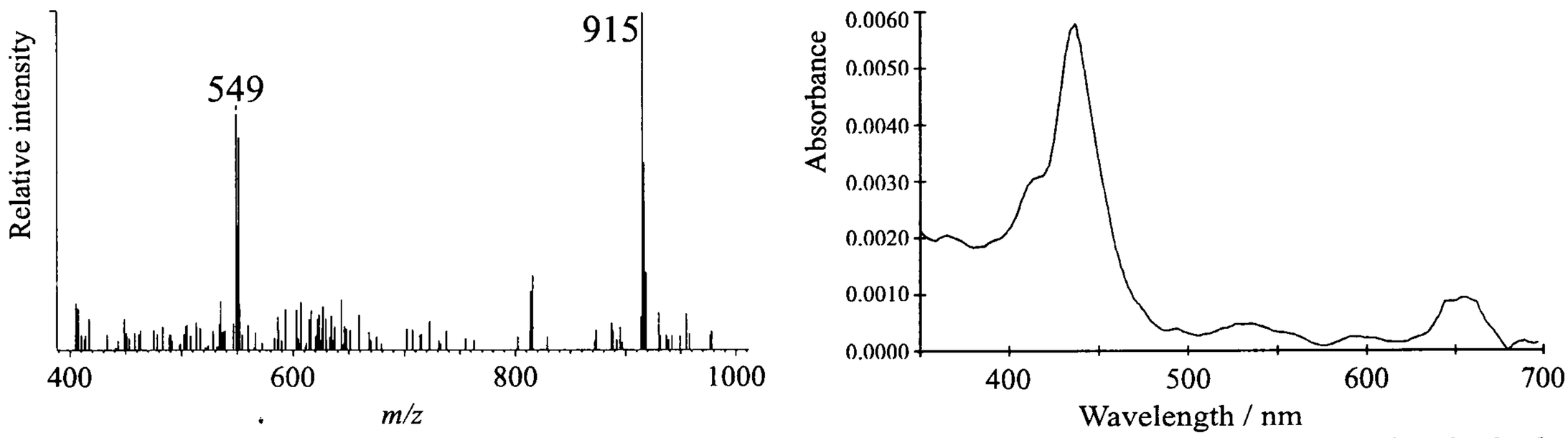


Figure 2-31. *Mass and electronic spectra of SCE peak* ***a*** *(single pyrophaeophorbide b ester $MH^+ = 915$).*

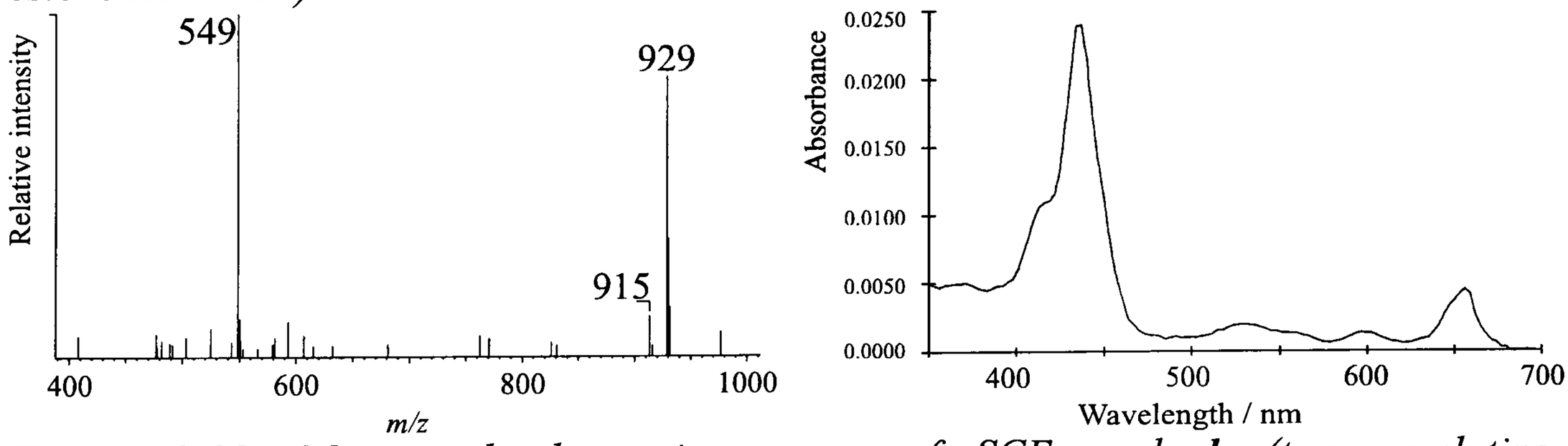


Figure 2-32. *Mass and electronic spectra of SCE peak* ***b*** *(two co-eluting pyrophaeophorbide b esters $MH^+ = 915$ and 929).*

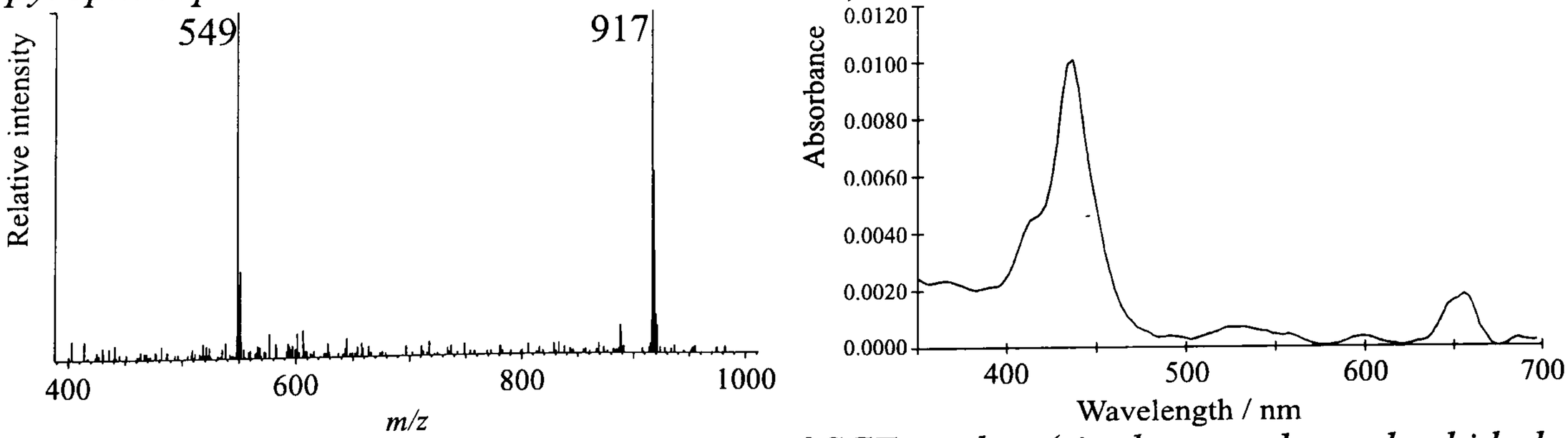


Figure 2-33. *Mass and electronic spectra of SCE peak* ***c*** *(single pyrophaeophorbide b ester $MH^+ = 917$).*

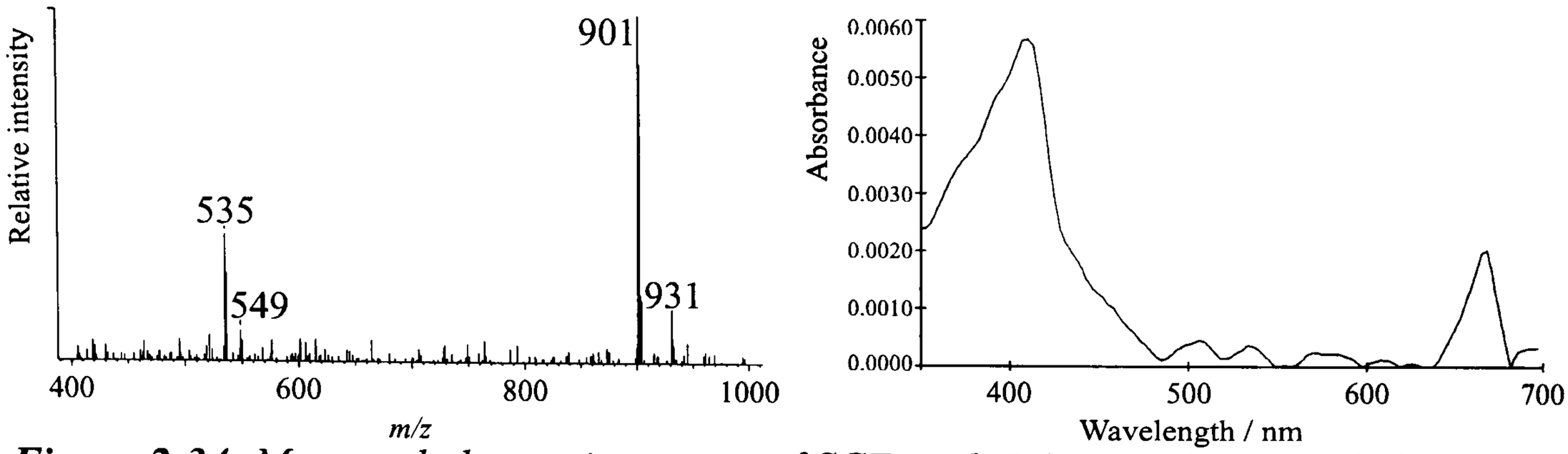


Figure 2-34. *Mass and electronic spectra of SCE peak* ***d*** *(1 pyrophaeophorbide b ester MH^+ = 931 and 1 pyrophaeophorbide a ester MH^+=901).*

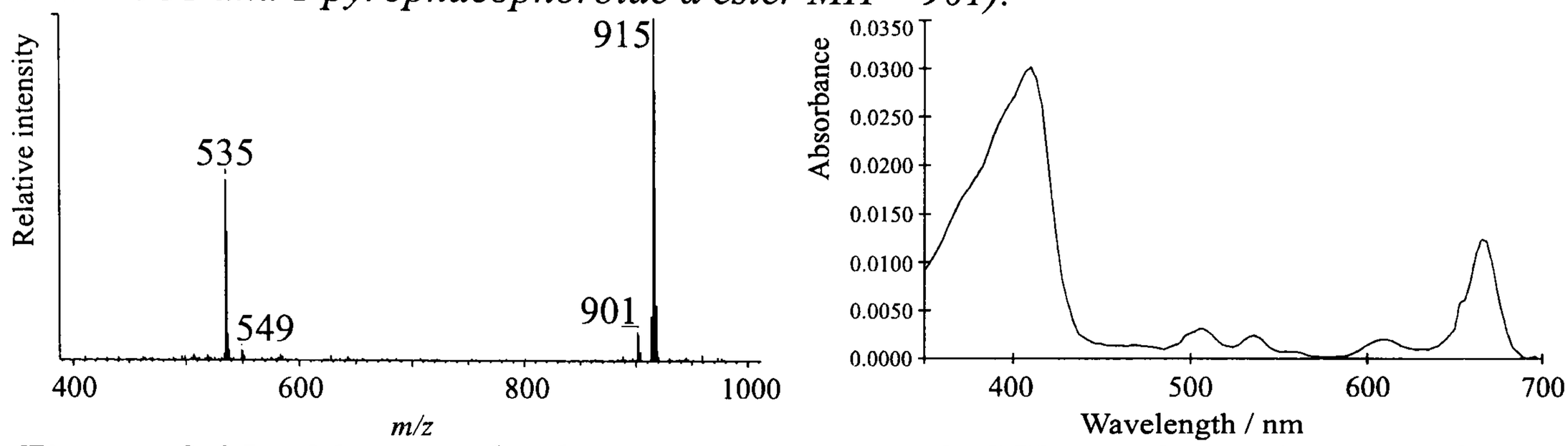


Figure 2-35. *Mass and electronic spectra of SCE peak* ***e*** *(two co-eluting pyrophaeophorbide a esters MH^+ = 901 and 915).*

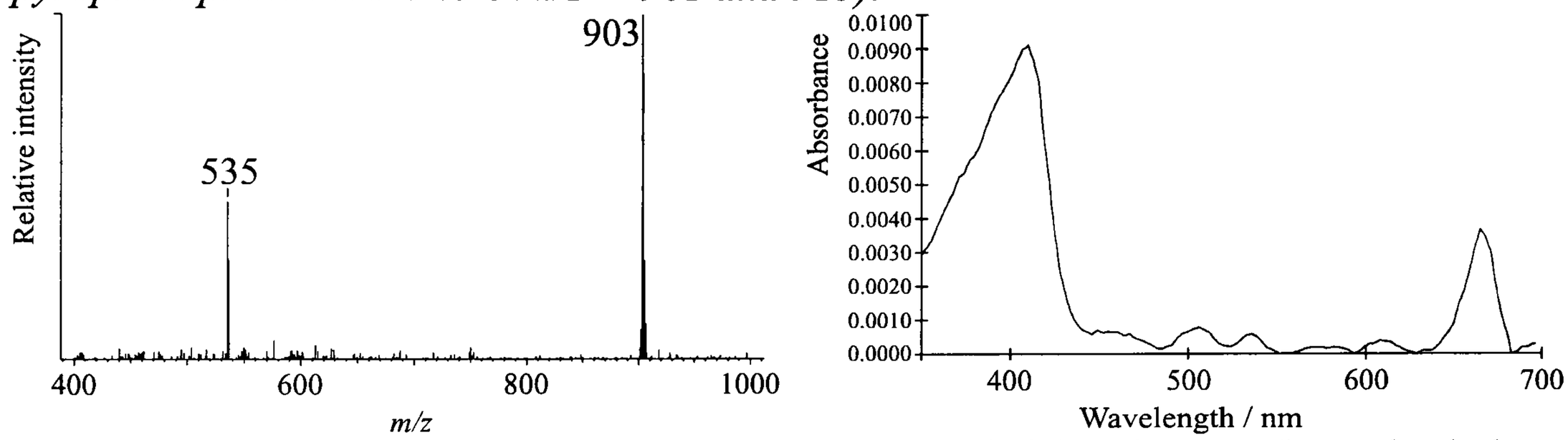


Figure 2-36. *Mass and electronic spectra of SCE peak* ***f*** *(single pyrophaeophorbide a ester MH^+ = 903).*

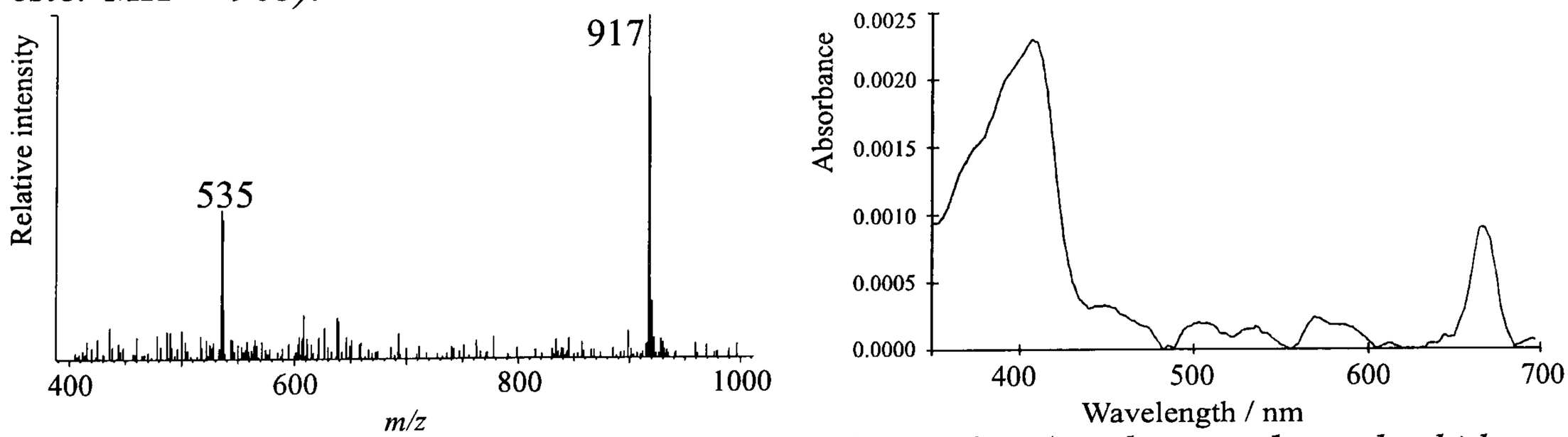


Figure 2-37. *Mass and electronic spectra of SCE peak* ***g*** *(single pyrophaeophorbide a ester MH^+ = 917).*

2.4.4. Free Sterols

2.4.4.1. Pre-Starved Copepods

The sterols of *C. helgolandicus* which had been allowed to empty their gut in filtered seawater for 24 h were identified by GC-MS (fig. 2-38) comparison of the spectra (fig. 2-39) with those of standards and literature data. The distribution was dominated by cholest-5-en-3β-ol (cholesterol; peak 2, **A1**) the most common zooplankton sterol (e.g. Gagosian *et al.*, 1981; Corner *et al.*, 1986; Volkman, 1986) which is often present in greater than 90% abundance, with the difference usually being made up of cholesta-5,24-dien-3β-ol (desmosterol; peak 4, **A2**) (e.g. Goad, 1978). The other abundant component, cholesta-5,22-dien-3β-ol (peak 1, **A3**) has recently been reported to be present in both *C. finmarchicus* and its faecal pellets after it had been allowed to graze on an algal diet known not to contain this sterol (Grice *et al.*, 1998). The fourth minor component was 5α-cholestan-3β-ol (peak 3, **C1**) and there was no indication of any C_{28} or C_{29} sterols normally associated with phytoplankton confirming that the copepod guts had been cleared.

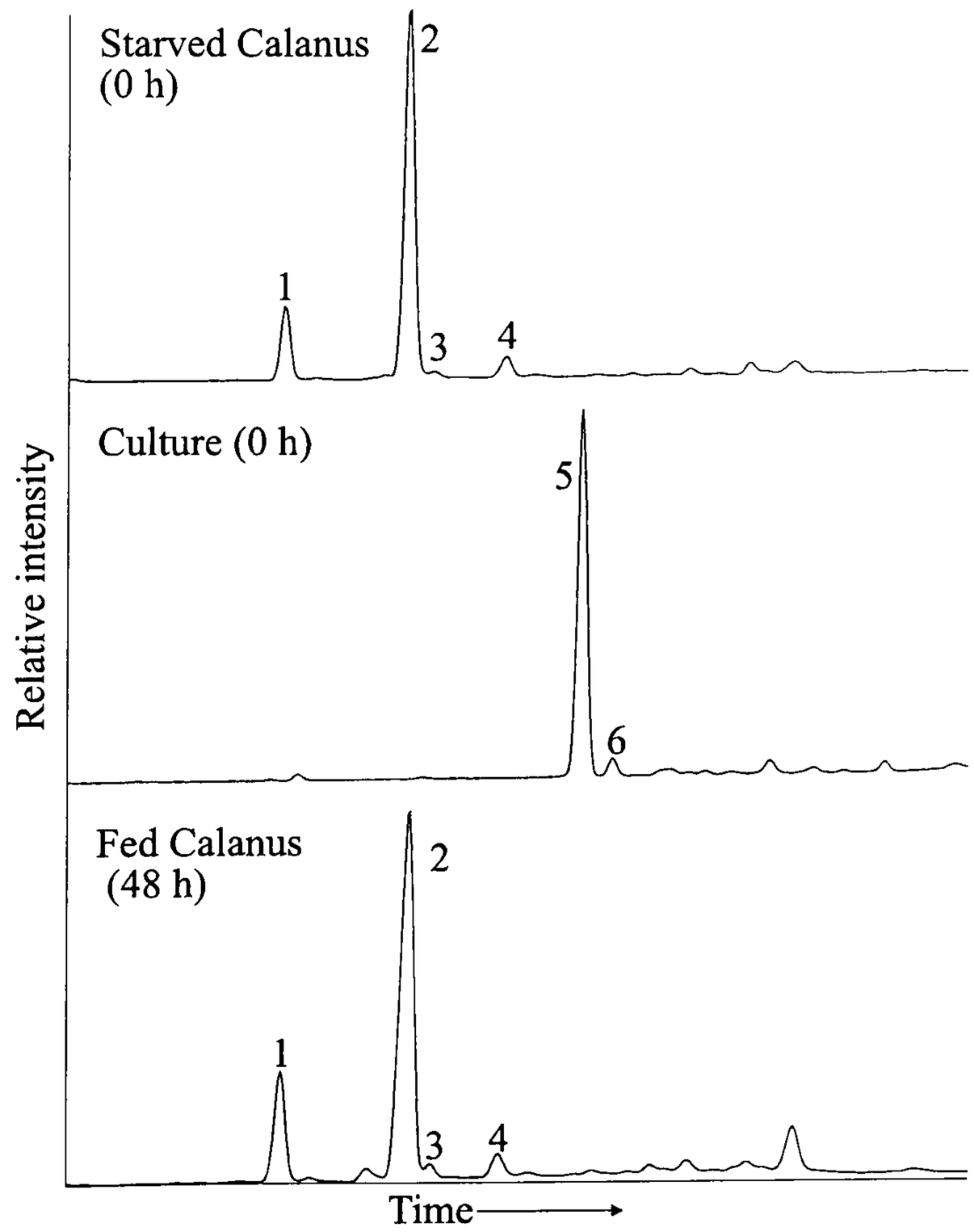


Figure 2-38. *Partial GC-MS RIC traces of free sterols (as TMSi ethers) in pre-starved copepods, algal culture and fed copepods. (Unlabelled peaks are non-sterol).*

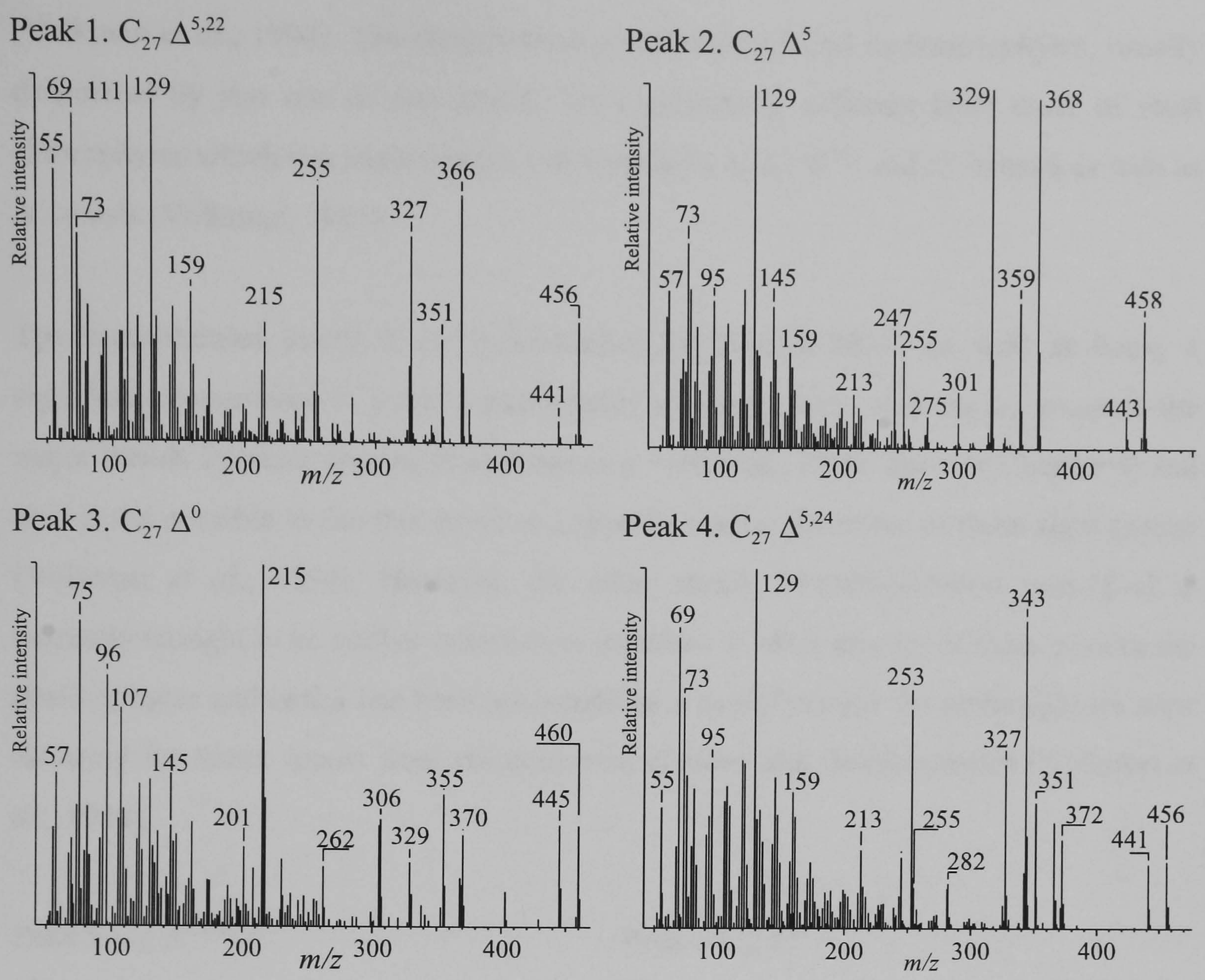


Figure 2-39. *Mass spectra of pre-starved C. helgolandicus sterols (TMSi ethers).*

2.4.4.2. Algal Culture

The algal culture contained only two C_{28} sterols in identical abundance at the commencement of feeding and after 24h (fig. 2-38). The distribution is dominated by 24-methylcholesta-5,24(28)-dien-3β-ol (Peak 5, **A4**), the less abundant component being 24-methylcholest-5-en-3β-ol (Peak 6, **A5**). The mass spectra are shown in Figure 2-40. The occurrence of only these two sterols in significant abundance is qualitatively similar to the results of Lin *et al.* (1982) who found them to be present in *T. suecica* in roughly equal abundance with a trace contribution (i.e. < 1%) from cholest-5-en-3β-ol; 24-methylcholesta-5,24(28)-dien-3β-ol was also found to be the most abundant sterol in two other species of prasinophyte and a major component in two others (Volkman *et al.*, 1994). The minor component 24-methylcholest-5-en-3β-ol also appears to be a common feature in prasinophyte sterol distributions, sometimes being the only significant sterol present. Also, the C_{29} sterol 24-ethylcholesta-5,24(28)Z-dien-3β-ol (**A7**) has been reported to be the most abundant sterol in other non-*Tetraselmis* spp. prasinophytes

(Volkman *et al.*, 1994). The simple sterol distributions found in prasinophytes, usually dominated by just one or two sterols, are significantly different from those of most chlorophytes which can show complex distributions of Δ^7, $\Delta^{7,22}$ and $\Delta^{5,7}$ sterols as well as Δ^5 sterols (Volkman, 1986).

The diunsaturated sterol 24-methylcholesta-5,24(28)-dien-3β-ol, as well as being a significant component in some prasinophytes as shown here, also occurs amongst the major sterols in many species of diatoms (e.g. Volkman, 1986; see also Chapter 4) and so it is not possible to use this sterol as a specific marker for either of these algal groups (Volkman *et al.*, 1994). However, the other sterol, 24-methylcholest-5-en-3β-ol is currently thought to be neither common or abundant in other species of algae besides the prasinophytes and hence has been postulated as a useful marker for prasinophytes after allowing for minor inputs from chlorophytes, diatoms and dinoflagellates (Volkman *et al.*, 1994).

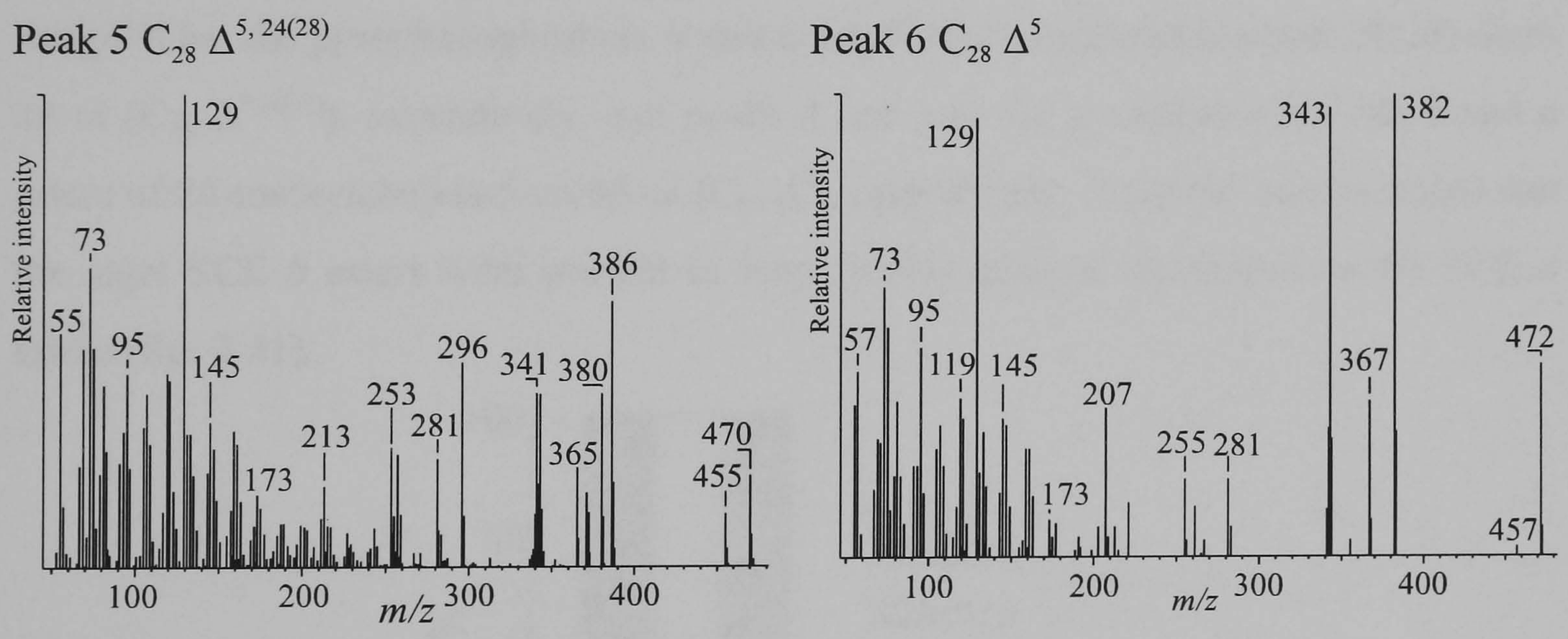


Figure 2-40. *Mass spectra of T. suecica sterols (TMSi ethers).*

2.4.4.3. Fed Copepods

After feeding the sterols present in the animals were the same as those in the pre-starved animals, although there were slight changes in the relative abundances. Particularly notable is a significant drop in the abundance of cholesta-5,24-dien-3β-ol (peak 4, **A2**) relative to the two major sterols. There was no indication of either of the algal sterols, suggesting that these sterols were either efficiently assimilated or excreted.

2.4.4.4. Comparison of Free and SCE Sterol Relative Abundance

As the sterols in the animals and alga are distinct, the relative abundance of the free components in the two pools are considered separately. The SCE fraction shows some degree of co-elution of certain components (see section 2.4.3; fig. 2-30) so it was not possible to measure the relative abundance of each of the SCE sterol components using the HPLC absorbance chromatograms and there was insufficient material to attempt to hydrolyse the total SCE fraction for analysis of the sterols by GC-MS. The components could however be measured separately using the area of the MH^+ peaks in the LC-MS mass chromatograms.

Algal sterols

The culture contained a di- and monounsaturated C_{28} sterol which, when esterified to pyrophaeophorbide *a,* would produce MH^+ of *m/z* 915 and 917 (*cf. m/z* 929 and 931 for pyrophaeophorbide *b*) and two SCEs *a* and two SCEs *b* were found in the faecal pellets with the appropriate MH^+ (see section 2.4.3). Therefore peaks b and e (fig. 2-30) are assigned as the pyrophaeophorbide *b* and *a* esters of 24-methylcholesta-5,24(28)-dien-3β-ol (C_{28} $\Delta^{5,24(28)}$), respectively, and peaks d and g as the pyrophaeophorbide *b* and *a* esters of 24-methylcholest-5-en-3β-ol (C_{28} Δ^{5}), respectively. It should also be noted that the algal SCE *b* esters were present in very similar relative abundance as the SCE *a* esters (fig. 2-41).

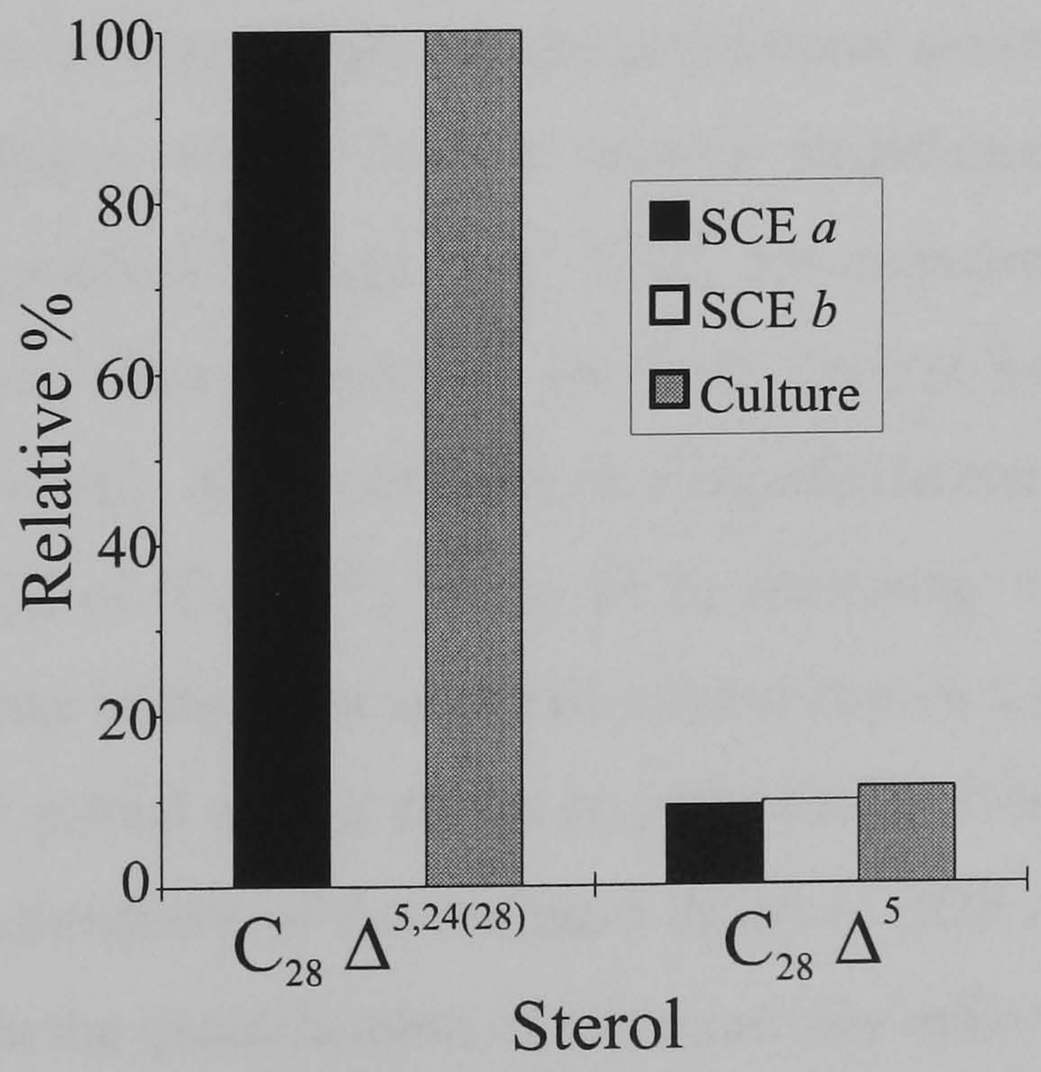


Figure 2-41. *Relative % of algal sterols in SCE a, SCE b and culture.*

It is also clear from Figure 2-41 that the pellet SCE sterols are present in the same relative abundance as the free sterols in the algal culture so both of the ingested algal sterols are converted to SCEs with the same efficiency of esterification.

Animal sterols

The two di- and one monounsaturated C_{27} sterols and the C_{27} stanol, when esterified to pyrophaeophorbide *a*, would produce MH^+ ions of *m/z* 901, 903 and 905, respectively (*cf.* *m/z* 915, 917 and 919 for pyrophaeophorbide *b*). The faecal pellet SCEs (fig. 2-30) show the presence of three C_{27} SCEs *a* and three C_{27} SCEs *b*, which could only be produced from sterols in the animals, the MH^+ values of which match those expected. The stanol, present in only trace amounts, would probably be below detection limits as SCEs in the pellet extract and ions of *m/z* 905 and 919 were not observed. The assignment of the two C_{27} diunsaturated sterol moieties as SCEs is based on the observation that for SCE esterified sterols containing the same number of carbon atoms, components with double bonds at C-5 and C-24 elute before sterols with double bonds at C-5 and C-22 (e.g. King and Repeta, 1991). Therefore, the diunsaturated C_{27} sterol moieties in SCE peaks a and d (fig. 2-30) are assigned as cholesta-5,24-dien-3β-ol (**A2**) and in peaks b and e (fig. 2-30) as cholesta-5,22-dien-3β-ol (**A3**). Components were normalised to cholesta-5,22-dien-3β-ol (C_{27} $\Delta^{5,22}$; Fig. 2-42) since cholesterol and its precursor cholesta-5,24-dien-3β-ol (C_{27} $\Delta^{5,24}$) are required by the animal whereas the C_{27} $\Delta^{5,22}$ sterol is thought to be a by product of the phytosterol assimilation pathway; Grice *et al.*, 1998). Comparison of the SCE *a* relative abundance with the free sterol abundance in the pre-starved animals (fig. 2-42; for convenience, distributions are related to the shows a close correlation for both cholest-5-en-3β-ol (C_{27} Δ^5) and cholesta-5,22-dien-3β-ol (C_{27} $\Delta^{5,22}$), but there is a significant increase in the abundance of cholesta-5,24-dien-3β-ol (C_{27} $\Delta^{5,24}$) in the SCE, indicating increased production of this SCE relative to those of the other available animal sterols. Comparison of the SCEs *b* with the pre-starved animal sterols shows broadly similar trends although there is a slight decrease in the abundance of the cholest-5-en-3β-ol SCE *b* which may simply be caused by inaccuracy in the quantification of this relatively minor peak (see fig. 2-30).

The fed animal sterols show a significant drop in the abundance of cholesta-5,24-dien-3β-ol relative to the pre-starved animals and hence an even greater difference between the fed animals and the SCEs (fig. 2-42).

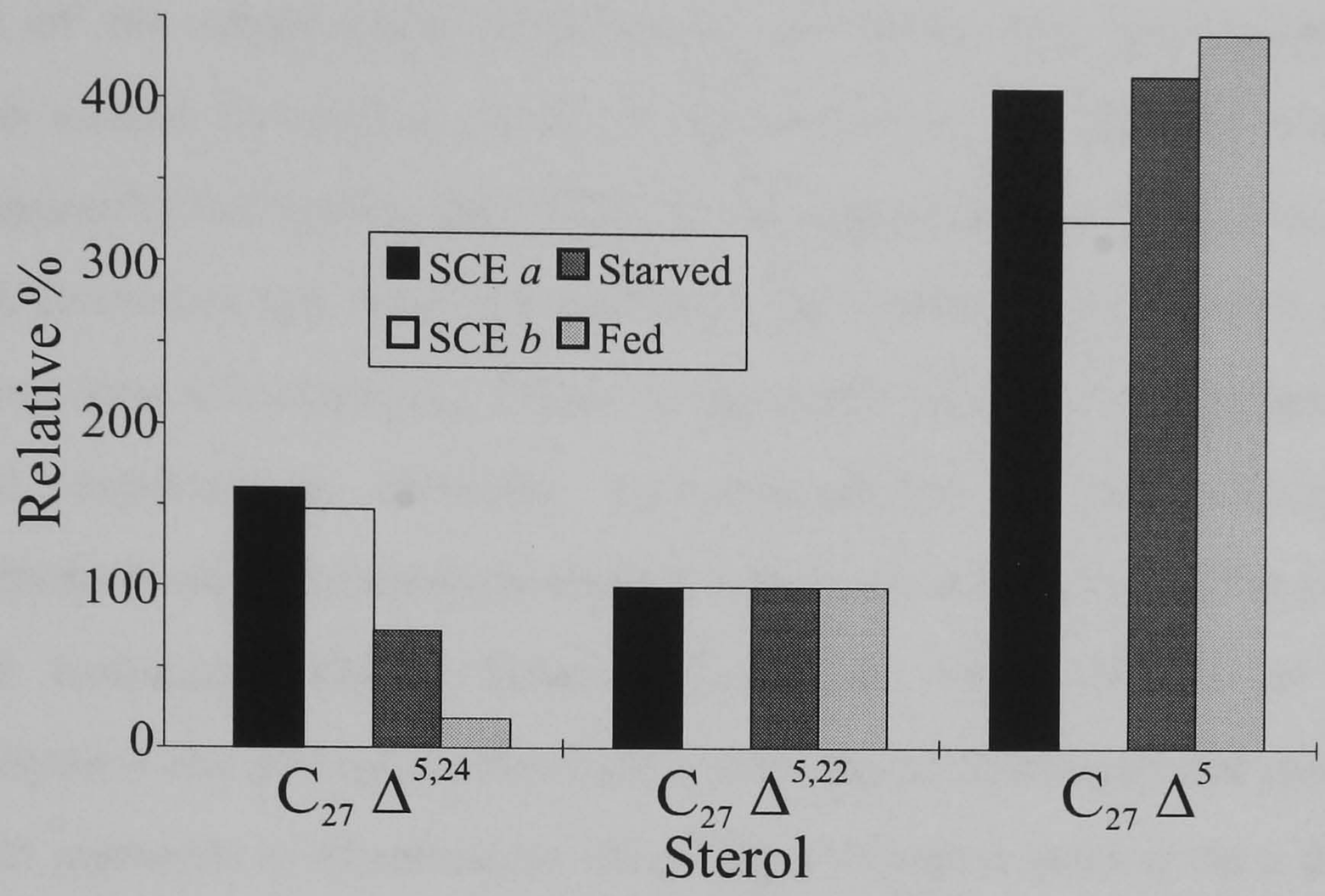


Figure 2-42. *Relative % of animal sterols in SCEs a, SCEs b, starved and fed copepods.*

Table 2-3 summarises the results for the SCEs in the large scale prasinophyte experiment including sterol assignments.

Peak*	MH^+	other ions	λ_{max} /nm	Assignment	Structure
a	915	549	434,530,599,656	pyrophaeophorbide b cholesta-5,24-dien-3β-ol ester	**XX-A2**
b	915	549	434,530,599,656	pyrophaeophorbide b cholesta-5,22-dien-3β-ol and 24-methyl	**XX-A3** **XX-A4**
	929			cholesta-5,24(28)-dien-3β-ol esters	
c	917	549	434,530,599,656	pyrophaeophorbide b cholest-5-en-3β-ol ester	**XX-A1**
d	931	549	410,506,536,608,665	pyrophaeophorbide b 24-methylcholest-5-en-3β-ol and	**XX-A5**
				pyrophaeophorbide a cholesta-5,24-dien-3β-ol	**X-A2**
	901	535			
e	901	535	410,506,536,608,665	pyrophaeophorbide a cholesta-5,22-dien-3β-ol and 24-methyl	**X-A3** **X-A4**
	915			cholesta-5,24(28)-dien-3β-ol esters	
f	903	535	410,506,536,608,665	pyrophaeophorbide a cholest-5-en-3β-ol	**X-A1**
g	917	535	410,506,536,608,665	pyrophaeophorbide a 24-methylcholest-5-en-3β-ol	**X-A5**

* see fig. 2-30

Table 2-3. *SCE data from large scale T. suecica experiment.*

2.5. DISCUSION

2.5.1. Pigments

The results of the small scale experiments, involving two chlorophytes and one prasinophyte extend the earlier study of Harradine *et al.* (1996b) with a diatom (Bacillariophyta) by confirming that SCEs are produced during zooplankton herbivory. Hence, SCE formation has been demonstrated for members of a second of the major algal divisions (the Chlorophyta). Other components observed in the products of the small scale experiments included pyrophaeophytin *a*, pyrophaeophorbide *a*, pyrophaeophytin *b* and pyrophaeophorbide *b* which are common grazing products (e.g. Vernet and Lorenzen, 1987a; Head and Harris, 1992; Otsuki *et al.*, 1993). Pyrophaeophytin *a* and pyrophaeophorbide *a* were found to account for more than 70% of total pellet pigments in experiments involving a copepod grazing on a diatom (Head and Harris, 1992) and also were the most abundant products observed by Harradine *et al.* (1996b) in the preliminary SCE study. Undegraded chl *a* and *b* are often detected in faecal pellets, indicating that some material passes through the guts of zooplankton unchanged and suggesting assimilation efficiencies lower than ingestion rates under certain feeding conditions. Downs and Lorenzen (1985) found that 5-7% of copepod faecal pellet pigments consisted of undegraded chl. The extent of chl degradation has been shown to depend on both long-term (*in situ*) and short term feeding history (e.g. Penry and Frost, 1991; Head, 1992) with animals which have been starved for up to 12 h prior to commencement of feeding degrading significantly higher proportions of chl than animals which have been starved for 24 h (Lopez *et al.*, 1988 and references therein). Unaltered carotenoids have also been found in copepod faecal pellets suggesting that they are not extensively degraded during passage through the copepod gut (Nelson, 1989).

SCEs *b* have been reported in two marine environments during the course of this work (Cariou-Le Gall *et al.*, 1998; Kowalewska *et al.*, 1999). Production of steryl chlorin esters containing the pyrophaeophorbide *b* moiety has now been confirmed for the first time in a feeding experiment. It is expected therefore, that where Chlorophyta are an

abundant component of the natural phytoplankton assemblage, SCEs *b* will be an important component of the of zooplankton faecal pellet pigment signatures and ultimately provide a significant sedimentary sink for chl *b*.

Purpurin-18-phytyl ester (**XIVa**), although absent from the culture and control, was present in the faecal pellets; this suggests that its occurrence is a result of herbivory. However, it was found to be a minor component in the culture, control and pellets in an experiment involving a diatom so it appears that it was probably present in this experiment in the culture but was below detection limits. This will be discussed in more detail in Chapter 4.

The possible occurrence of methoxylactone phaeophytin *a* (**XXXIV**) in the pellets is unexpected due to the lack of exposure to a methylating agent during sample extraction and work-up.

A series of highly non-polar, high molecular weight components were identified in the faecal pellet extracts of the three small scale feeding experiments. However, there was insufficient material to allow full characterisation of these novel components and they were surprisingly not observed in the pellet extract from the large scale repeat of the prasinophyte experiment; neither were they detected in the products of the other feeding experiments carried out during this work (Chapters 3-6).

2.5.2. Sterols

The SCE fraction (pyrophaeophorbide *a* and pyrophaeophorbide *b* esters) was found to contain both of the available algal sterols in the same relative abundance at which they occur in the algae, providing further circumstantial evidence to support the suggestion that SCEs accurately reflect the sterol distribution of the original phytoplankton population (King and Repeta, 1991, 1994); however, other results suggest that this is not always be the case (Chapters 3-5). The SCEs also contained the major sterols present in the animal, so SCEs can represent inputs from the heterotrophic community. Excluding C_{27} sterols from the SCE sterol distribution as suggested by Repeta (1995) would be

valid in this situation; however, as many algal species are known to contain cholest-5-en-3β-ol as a major sterol (e.g. some dinoflagellates) this could bias the interpretation of the phytoplankton signature in natural samples.

It is now well established that crustaceans are incapable of *de novo* sterol biosynthesis as are the other major class of Arthropoda, the insects (e.g. Goad, 1978, 1981) and are therefore dependent on a dietary sterol source. Insects have long been known to perform dealkylation of C-24 substituted C_{28} and C_{29} phytosterols in order to produce cholest-5-en-3β-ol and the ability of crustaceans to perform the same process has also been substantiated (reviewed by Goad, 1978; and see references therein). The mechanism of C_{24} dealkylation in crustaceans has not yet been fully established but assuming it is the same as that operating in insects, this would explain the appearance of cholesta-5,24-dien-3β-ol (**A2**) in many crustaceans as it is produced as an intermediate in the process and then undergoes reduction to cholest-5-en-3β-ol (see fig. 2-43).

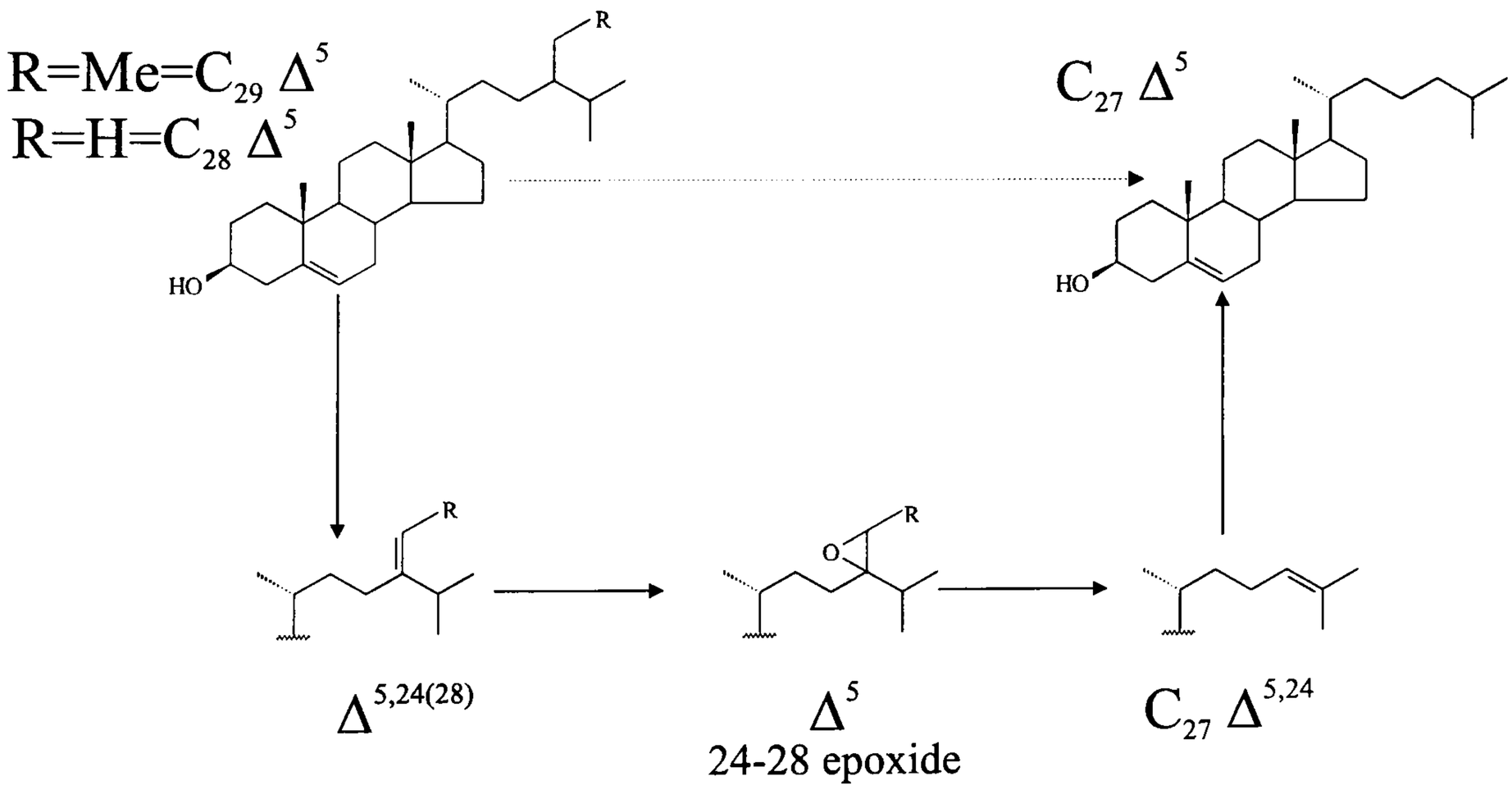


Figure 2-43. *Mechanism of sterol C_{24} dealkylation operating in insects and probably crustaceans (Goad, 1978, 1981; Svoboda and Feldlaufer, 1991).*

As there was no cholest-5-en-3β-ol in the alga it is possible that the reduction in cholesta-5,24-dien-3β-ol (desmosterol) observed in the fed animals relative to the starved animals could be due to utilisation of this sterol for the production of cholest-5-en-3β-ol, although some cholesterol was excreted in the pellets in the form of SCEs and copepods have previously been reported to excrete as much as 4 ng cholesterol/pellet. It has been suggested that a high abundance of cholest-5-en-3β-ol in copepod pellets could represent its production from phytosterols in excess of nutritional needs (Prahl *et al.*,

1984). Based on the reports concerning insect phytosterol dealkylation it would seem likely that in this case the copepod could have produced sufficient cholesterol for its needs from the ingested algal sterols and the reduction in the desmosterol pool could reflect its use during the period of acclimatisation to the new food conditions when the copepods were transferred from the filtered seawater to the culture feeding concentration. Copepods have been shown to require time to become accustomed to new feeding regimes after being starved for 12-24 h prior to commencement of feeding (Lopez *et al.*, 1988 and references therein). Based on the close relationship between the relative abundance of the free and SCE algal sterols it seems likely that in this case both sterols underwent esterification with equal efficiency and that both sterols were also assimilated for the production of cholesterol with equal efficiently. It also seems likely therefore, that during grazing on an alga which does contain an abundance of cholest-5-en-3β-ol, that the reduction in the abundance of desmosterol in the SCEs relative to the starved animals would not be observed.

2.6. SUMMARY

SCE formation during zooplankton herbivory has been confirmed with three members (*D. tertiolecta, C. reginae* and *T. suecica*) of a major algal division (the Chlorophyta). The production of pyrophaeophorbide *b* steryl esters (SCEs *b*) has been confirmed for the first time in the laboratory when *C. helgolandicus* grazed on the prasinophyte *T. suecica*, thereby indicating that SCEs could be a major sink for chl *b* as well as chl *a*. The two algal sterols were incorporated into the SCE fraction in the same relative abundance as they occurred in the alga, indicating that in this case SCEs are a good indicator of the substrate alga. The three major animal sterols were also incorporated into the SCEs with some degree of alteration suggesting that SCEs are at least a good qualitative indicator of zooplanktonic herbivores as well as of phytoplankton.

Chapter 3

FEEDING EXPERIMENTS WITH HAPTOPHYTES

3.1. INTRODUCTION

3.1.1. The Haptophyceae

In order to investigate which other algal classes are involved in SCE production and to determine if any group or groups show a particular propensity for high levels of production it was decided to investigate one of the most important divisions of marine algae, the Haptophyta. This division is part of a group commonly referred to as the golden-brown flagellates, which consists of the Haptophyta and the Chrysophyta; however, as no studies involving species of Chrysophyta were undertaken during this work the present discussion is limited to the Haptophyta only.

The division Haptophyta consists of only a single class, the Prymnesiophyceae, which includes the coccolithophorids and scale-bearing golden-brown flagellates, containing around 75 genera and about 500 species (van den Hoek *et al.*, 1995). They are unicellular and are either flagellated or have a flagellated motile stage during their life cycle (Jeffrey and Vesk, 1997). The majority of Haptophyta are marine species with only a few freshwater species known (van den Hoek, 1995). They range in size from 2-100 μm but most are in the nanoplankton (2-20 μm) range and together with the picoplankton are responsible for the largest proportion of primary production in the open ocean. They are, however, also common constituents of phytoplankton communities in nearshore and inshore waters.

The coccolithophorids are the best known group being covered in calcite scales (coccoliths) which often have intricate patterns specific to each species which can be readily identified using electron microscopy. The function of the coccoliths is still not clearly understood but some possibilities include a skeletal function, a protective device for the cell membrane, a light scattering function and a mechanism for removing calcium deposited in association with a bicarbonate photosynthesis (Jeffrey and Vesk, 1997). Coccolithophorids are mainly warm water species, showing the greatest diversity in tropical and subtropical waters; however, some species such as *Emiliania huxleyi* have both warm and cold water forms and this species has the widest geographical range

of all known haptophytes. *Emiliania huxleyi* is a bloom-forming species which can be clearly seen using remote sensing over wide areas due to light scattering of detached coccoliths which have negligible sinking rates and so remain in the surface layers (Aiken *et al.*, 1992). It is thought to be the most important producer of biogenic calcium carbonate, thus playing a major role in the global CO_2 budget. After death the coccolithophorids sink to the bottom of the ocean where they form calcareous deposits in abyssal sediments. They can also be transported to the sea floor in zooplankton faecal pellets (e.g. Harris, 1994).

The species which do not have coccospheres are covered in several layers of organic scales made up of polysaccharides and which show a characteristic radial ridge pattern. These forms are less well known than the coccolithophorids as they are destroyed by standard preservatives (Jeffrey and Vesk, 1997).

3.1.2. Photosynthetic Pigments

The Haptophyceae show significant diversity in their pigment signatures although they are similar to the most commonly observed distributions in the Bacillariophyceae (see Chapter 4). Jeffrey and Wright (1994) examined 29 species of cultured haptophytes and found four distinct pigment groupings. The first group (Type 1) contain chl *a*, chls c_1 (**III**) and c_2 (**IV**), β,β-carotene (**XXXV**), fucoxanthin (**XXVI**), diadinoxanthin (**XXXVI**), and diatoxanthin (**XXXVII**). Type 2 is similar except that chl c_1 is replaced with c_3 (**V**). Type 3 contains chl *a*, chls c_2 and c_3, a phytylated chl *c*-like derivative (**XXXVIII**; e.g. Jeffrey *et al.*, 1997), with 19'-hexanoyloxyfucoxanthin (**XXXIX**) the dominant carotenoid and in Type 4 19'-hexanoyloxyfucoxanthin dominates with chls and other carotenoids present in varying abundance.

3.2. FEEDING EXPERIMENTS

To investigate the production of SCEs during herbivorous grazing on haptophytes, a series of feeding experiments were set up involving starved Stage V and females of *C. helgolandicus* grazing on individual cultures of three species with different

characteristics: *Coccolithus pelagicus*, *Isochrysis galbana*, and *Pleurochrysis* (previously *Crycosphaera*) *carterae*.

The first two experiments were performed using *C. pelagicus*, a large (12-18 μm) heavily armoured coccolithophorid widely occurring in the Northern hemisphere, and *I. galbana*, a smaller (5-6 x 3-4 μm) non-coccoid species which occurs in the northern hemisphere and Pacific. This species is also commonly used as food in marine culture. Subsequently, the third experiment using the coccolithophorid *P. carterae* (6-12 x 6-8 μm), a common species widely distributed around the temperate coasts of Europe (van den Hoek *et al.*, 1995), was performed after the first two experiments failed to demonstrate the production of SCEs.

As production of SCEs was only observed during the *P. carterae* experiment only the results from this experiment will be discussed in detail (but see Discussion). The structure of the experiment is identical to that of the *T. suecica* (prasinophyte) experiment (Chapter 2) and the samples and analysis protocols were as shown for the large scale prasinophyte experiment (fig. 2-1; Chapter 2).

3.3. RESULTS

3.3.1. Algal Culture

The HPLC chromatogram of the *P. carterae* extract (410 nm; fig. 3-1) shows no evidence of intact chlorophyll *a*, suggesting either high enzyme activity during extraction, insufficient precautions taken to minimise exposure to heat and light during extraction (however, this seems unlikely as the extraction protocol used was identical to that used for the *T. suecica* experiment in which both chls *a* and *b* were observed in high abundance), or that the culture had reached stationary phase and was beginning to undergo senescence before the experiment was carried out.

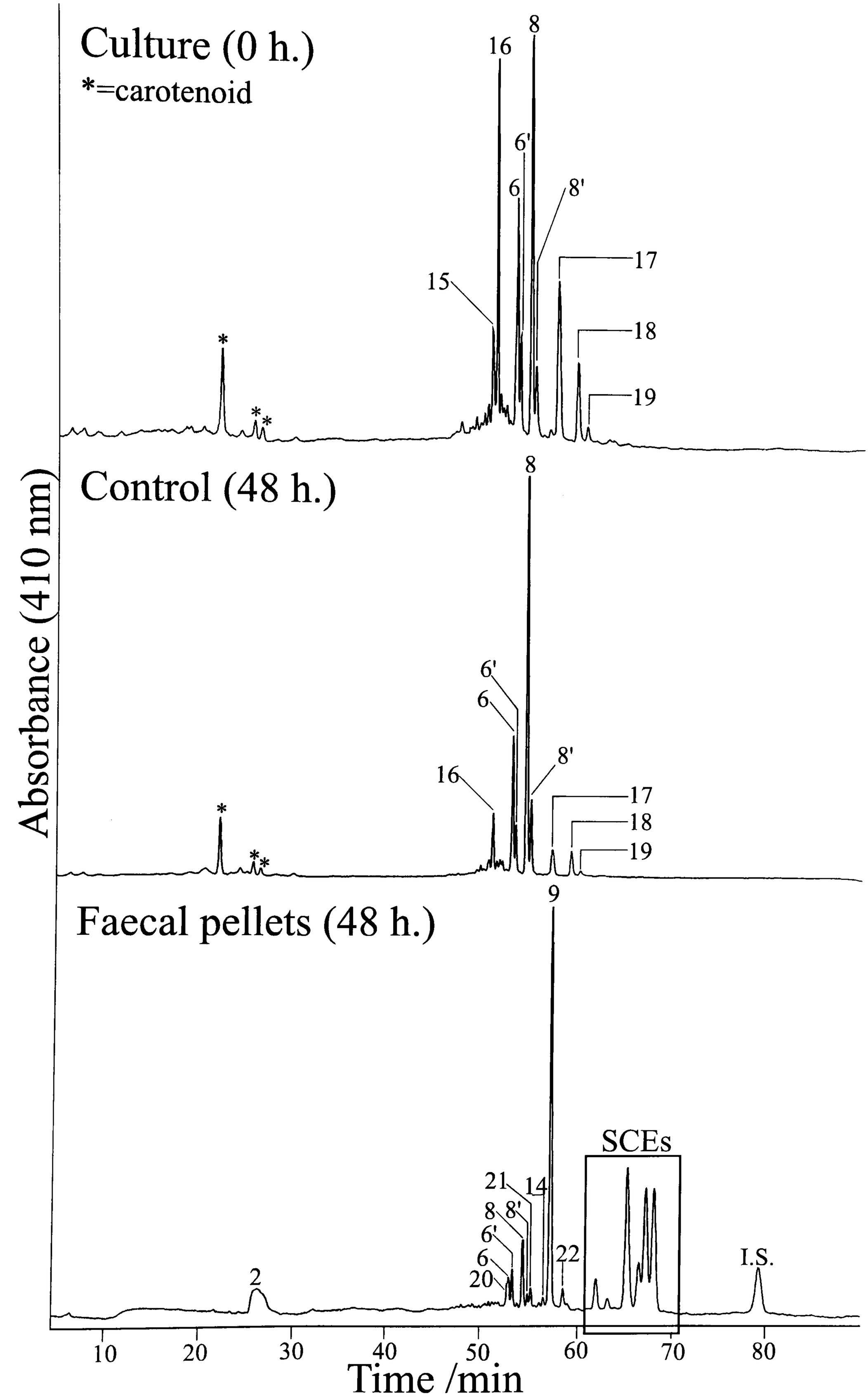


Figure 3-1. *HPLC chromatograms (410nm) from copepod feeding on P. carterae.*

The distribution is dominated by epimeric mixtures of phaeophytin *a* (peaks 8 and 8', **VIII**; see chapter 2 for mass and electronic spectra and Table 3-1) and C-13^2 hydroxyphaeophytin *a* (peaks 6 and 6', **XIII**). Also present are two peaks eluting prior to C-13^2 hydroxyphaeophytin *a*. The electronic spectrum of Peak 15 ($\lambda_{max.}$ 428, 542, 587, 623 and 668 nm) is similar to that of chl *a* although there is less structure on the blue side of the Soret band. The mass spectrum shows only one significant ion at *m/z* 577 which does not obviously correspond to a fragment of any known chl *a* allomer so this component could not be assigned and was not observed in any of the other feeding experiments. The mass and electronic spectral data for this and the other novel components observed in this experiment are summarised in Table 3-1.

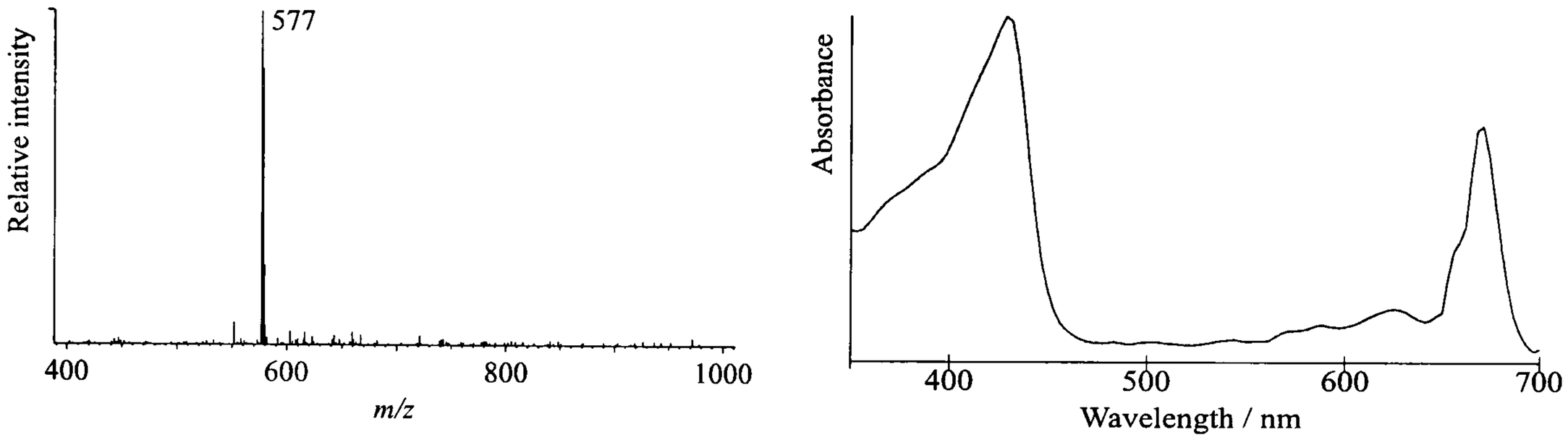


Figure 3-2. *Mass and electronic spectra of peak 15.*

Peak*	MH$^+$	other ions	λ_{max} /nm	Assignment	Structure
15		577	428,542,587,623,668	chl *a*-like	
16		907,705,664,649, 647,633,577	431,587,626,668	chl *a*-like	
17	921	903,687,669,643, 525,593,565	410,512,545,617,674		
18	905	627,567	410,482,512,545,620, 674		
19		627	410,479,509,545,617, 647,674		
20[1]		641,623,591	401,500,530,611,668		
21	811	(593),535	410,503,533,608,668	*pyrophaeophorbide a* $C_{20}H_{36}$ *ester*	***XXXX***
22	815		410,503,536,608,668	*pyrophaeophorbide a dihydrophytol ester or mesopyrophaeophytin a*	***XXXXI*** ***XXXXII***

* see fig. 3-1; [1] *cf.* Chapter 2, Peak 13 fig. 2-28; assignments in italics tentative

Table 3-1. *Pigment data for P. carterae experiment (NB. data for pigments described in chapter 2 not shown; see table 2-3).*

The electronic spectrum of peak 16 ($\lambda_{max.}$ 431, 587, 626 and 668 nm; fig. 3-3) is again similar to that of chl *a*. The mass spectrum is more complex than that of peak 15, but is still dominated by a single ion, *m/z* 647, with minor contributions from ions of *m/z* 705, 664, 633, 577 and a possible MH^+ of *m/z* 907. The base peak ion corresponds to that of C-15[1] hydroxy lactone chl *a* after loss of $C_{20}H_{38}$ suggesting the dioxygenated allomer (**XXX**) but this is disputed by the lack of a hypsochromic shift in the electronic spectrum observed for lactones (e.g. Woolley *et al.*, 1998); also the mass spectrum showed differences from that of C-15[1] hydroxy lactone chl *a* (fig. 2-13) identified in Chapter 2. Therefore this component could only be tentatively assigned as a possible oxygenated chl *a*-like component.

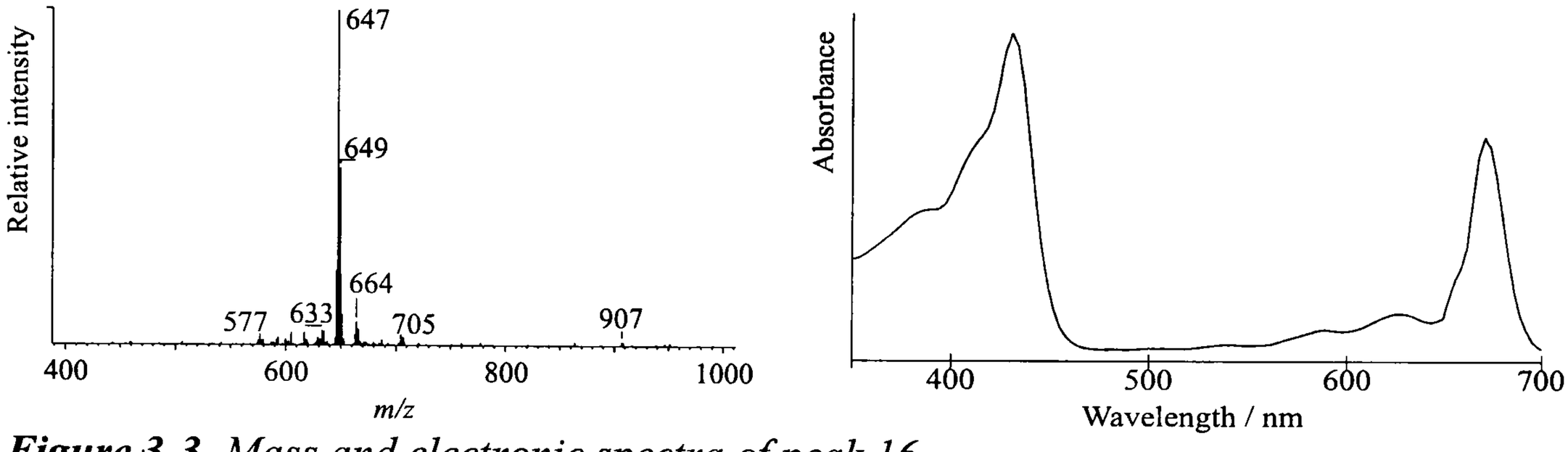


Figure 3-3. *Mass and electronic spectra of peak 16.*

The three peaks eluting after phaeophytin *a*' (peak 8') were unexpected highly non-polar components, two of which were not observed in any other feeding experiment. Peak 18, however, was observed co-eluting with a possible SCE *b* (fig. 2-9b, Chapter 2) in the faecal pellets from the small scale *T. suecica* experiment and was also present in a dinoflagellate experiment (see Chapter 5). The three components appear to be related, exhibiting identical electronic spectra which are qualitatively similar to that of pyrophaeophytin *a*, but with unusual bathochromic shifts in all absorbance maxima except the Soret band ($\lambda_{max.}$ 410, 479, 509, 545, 617, 674). The mass spectrum of peak 17 (fig. 3-4) shows a possible MH^+ at *m/z* 921, with the ion at *m/z* 903 suggesting loss of H_2O. The ion at *m/z* 643 presumably results from the loss of $C_{20}H_{38}$ (278 amu) from *m/z* 921 followed by loss of H_2O to give *m/z* 625. The fragment ion at *m/z* 593 (normally associated with the phaeophorbide *a* macrocycle) in this case seems more likely to originate from loss of MeOH from *m/z* 625 (suggesting that a carbomethoxy group is still present at the *m/z* 625 stage (*cf.* loss of MeOH from *m/z* 871 in phaeophytin *a*; Chapter 2). The MH^+ at *m/z* 921, 50 amu higher than that of phaeophytin suggests the

presence of more than one additional oxygen atom, but this is disputed by the retention time as oxidation typically leads to more polar products which would elute earlier. Therefore this component remains unassigned.

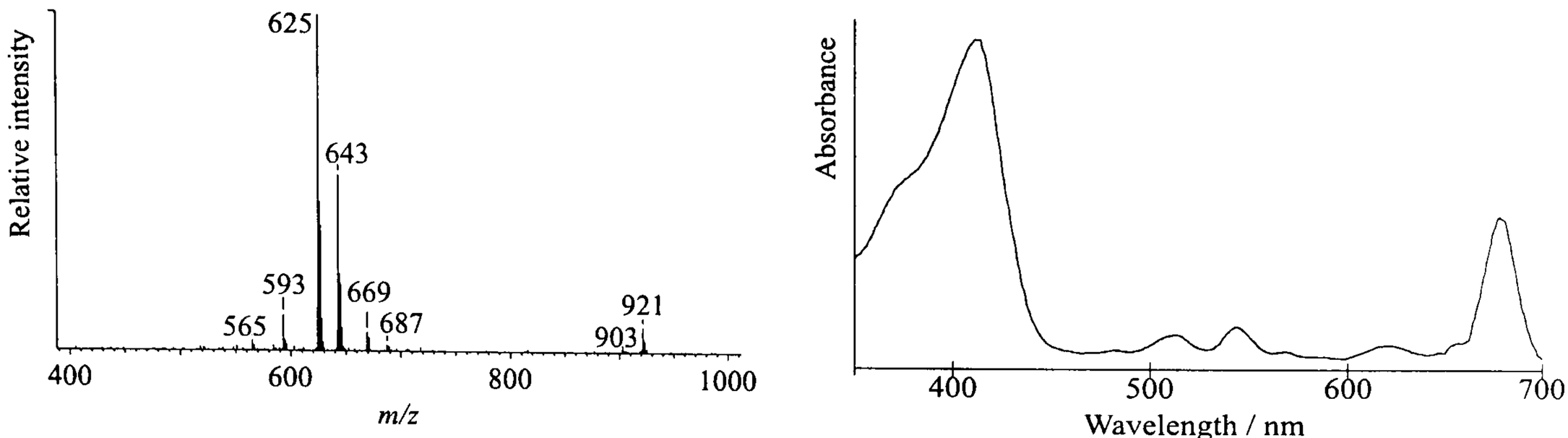


Figure 3-4. *Mass and electronic spectra of peak 17.*

The mass spectrum of peak 18 (fig. 3-5) is somewhat simpler, with MH^+ at *m/z* 905 also showing loss of $C_{20}H_{38}$ to give *m/z* 627 followed by loss of 60 mass units, presumably a C-13[2] carbomethoxy group. The ions at *m/z* 905 and 627, being two mass units higher than those for the equivalent fragmentation in peak 17. It is unlikely, however, that this component is the meso counterpart or ring opened counterpart of the *m/z* 903 fragment in peak 17 as the there would be clear differences between the electronic spectra of the two components which are not evident hence this component is also unidentified.

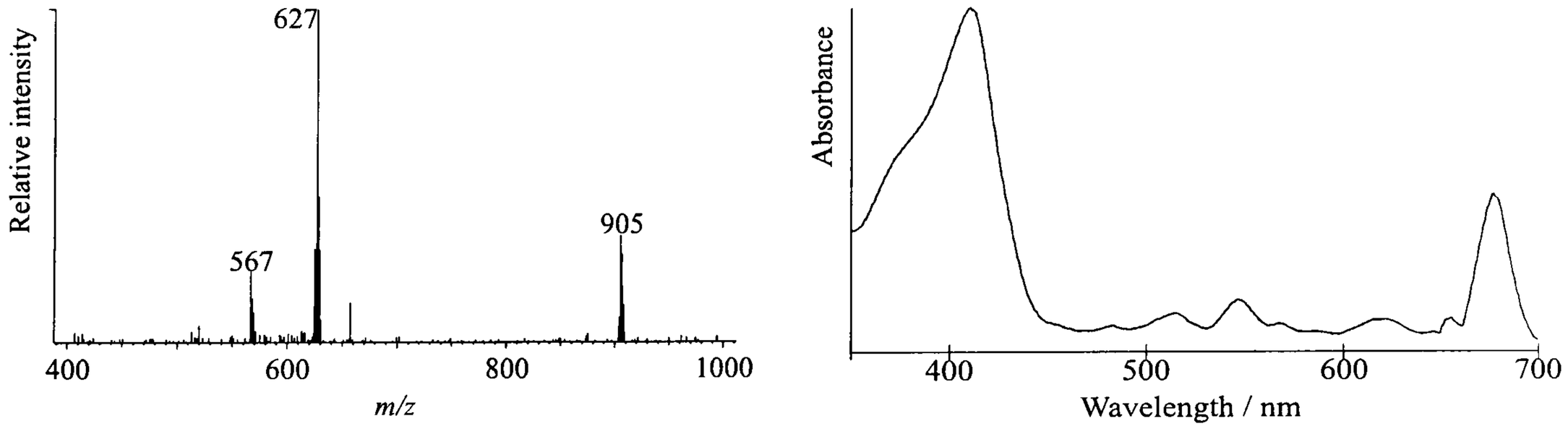


Figure 3-5. *Mass and electronic spectra of peak 18.*

The minor component, peak 19, shows only a single ion in its mass spectrum indicating a possible phaeophorbide-like equivalent derived from the phaeophytin-like peak 18, however, that being the case this component would be significantly more polar than peak 18 and would consequently elute earlier than peak 18. It seems likely therefore, that peak 19 is in fact an epimer of peak 18 and only the base peak is observed in the mass spectrum (fig. 3-6) due to the relatively low abundance of this component.

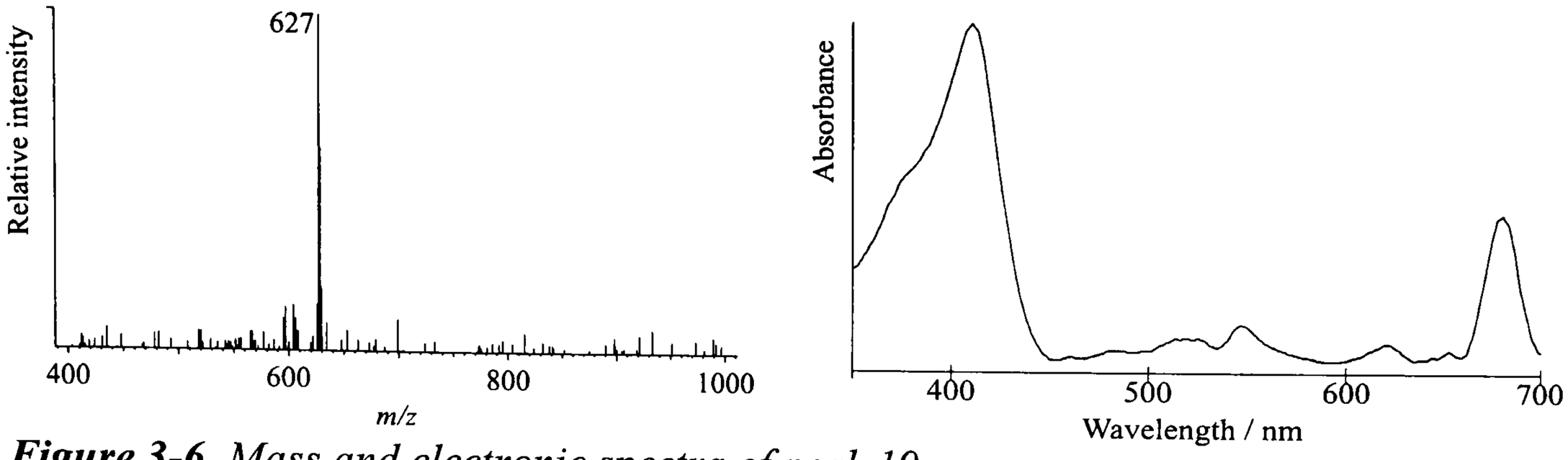


Figure 3-6. *Mass and electronic spectra of peak 19.*

A number of other chlorin-type components were also present in trace amounts showing only weak mass and electronic spectra, so are not considered to be significant components. The group of polar components eluting between 10 and 30 min are all carotenoids and were not investigated further. There was no indication of chlorophyllide *a* (**IX**) or phaeophorbide *a* (**VII**) in the culture.

3.3.2. Algal Control

The major pigments of the control (fig. 3-1) were similar to those of the fresh culture although the distribution was less complex. The differences may have arisen due to the use of glass fibre filters (GF/F) to collect the samples as they have been shown to cause pigment alteration due to their slightly acidic properties. The distribution is clearly dominated by phaeophytin *a* (peaks 8 and 8') with lower proportions of C-13^2 hydroxyphaeophytin *a* (6 and 6') and the 5 significant unknown components (peaks 15, 16, 17, 18 and 19). The carotenoid distribution was also similar to that of the culture.

3.3.3. Faecal Pellets

The distribution (fig. 3-1) is dominated by peak 9 identified as pyrophaeophytin *a* (**XI**; see Chapter 2) with a small poorly resolved peak (2) identified as pyrophaeophorbide *a* (**X**). Also present are lower quantities of phaeophytin *a* (8 and 8') and C-13^2 hydroxyphaeophytin *a* (6 and 6'). Peak 20, partially co-eluting with C-13^2 hydroxyphaeophytin *a* has an electronic spectrum ($\lambda_{max.}$ 401, 500, 530, 611 and 668 nm; fig. 3-7) which is similar to that of peak 13 (fig 2-10, Chapter 2) in the *T. suecica*-derived faecal pellet extract, showing hypsochromic shifts indicative of a lactone

structure. However, only one of the three major ions observed here (*m/z* 623) was present in the spectrum of peak 13 hence this peak can only be assigned as a phaeophytin *a* lactone-like component.

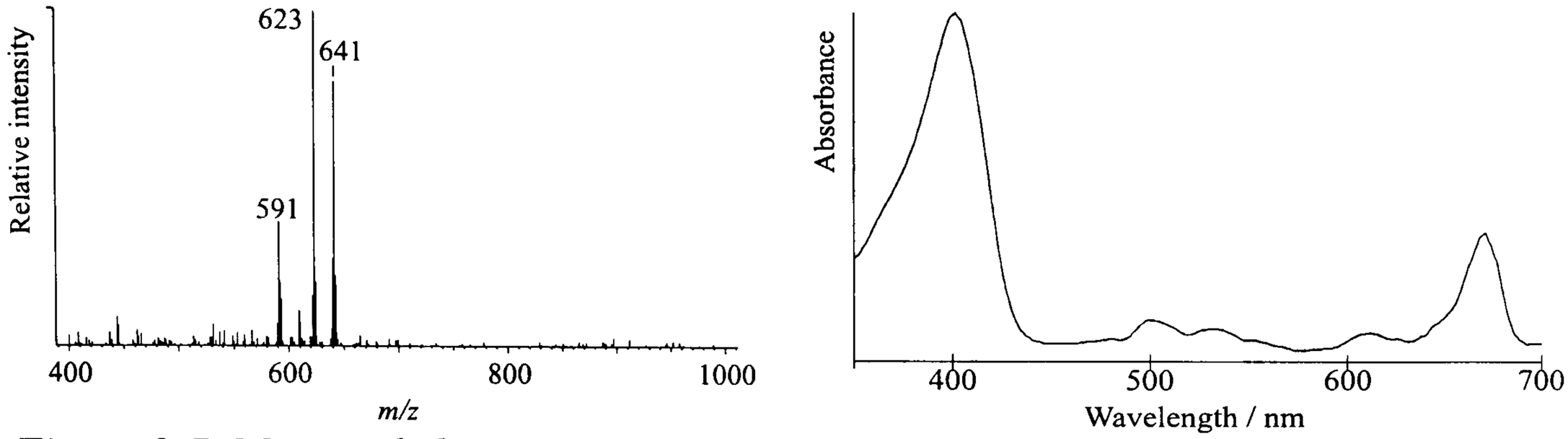


Figure 3-7. *Mass and electronic spectra of peak 20 (phaeophytin a lactone-like).*

Peaks 21 and 22 both have electronic spectra comparable with that of pyrophaeophytin *a* ($\lambda_{max.}$ 410, 503, 536, 608 and 668 nm). The mass spectrum of the more polar compound (peak 21; fig. 3-8) has MH^+ at *m/z* 811 with fragment ions of *m/z* 593 and 535, but the ion at *m/z* 593 is thought to be due to partial co-elution with peak 8'. Therefore the only genuine fragmentation is loss of 276 amu to give the ion at *m/z* 535, indicating the pyrophaeophorbide *a* moiety. This indicates that the esterifying alcohol is a C_{20} alcohol with two double bonds, possibly a dehydrophytol structure with the position of the second double bond being unknown (**XXXX**).

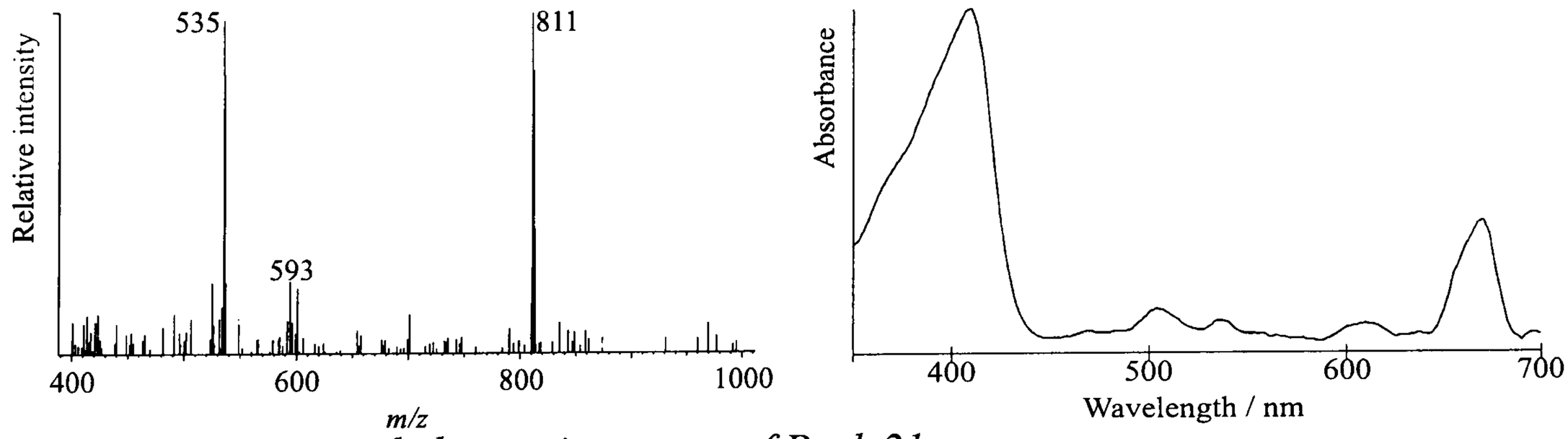


Figure 3-8. *Mass and electronic spectra of Peak 21.*

The second (peak 22) has a very simple mass spectrum (fig. 3-9) consisting only of a single ion at *m/z* 815. Again the electronic spectrum suggests the presence of the pyrophaeophorbide *a* moiety. Two possible structures for this component are a pyrophaeophorbide *a* dihydrophytyl ester (**XXXXI**) or mesopyrophaeophytin *a* (**XXXXII**). However, these components would be expected to show a fragment ion at *m/z* 535 or 537 respectively, which was not apparent.

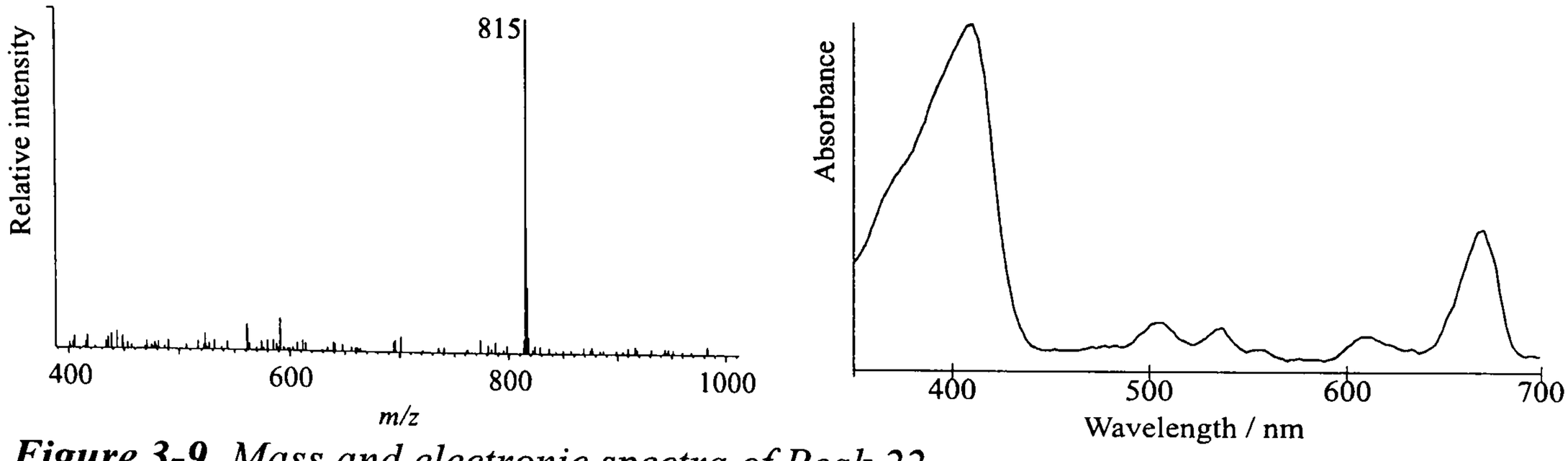


Figure 3-9. *Mass and electronic spectra of Peak 22.*

Also present is the minor component peak 14 assigned (*cf.* Chapter 2) as purpurin-18-phytyl ester (**XIVa**) based on retention time and the presence of the *m/z* 565 ion in the mass spectrum (*cf.* Naylor and Keely, 1998). There was no indication of any of the unknown components present in the culture and control samples (i.e. peaks 15-19).

The group of components in the SCE region consists of a total of 6 peaks, all with pyrophaeophorbide *a*-type electronic spectra ($\lambda_{max.}$ 410, 506, 536, 608 and 665 nm). Mass chromatography (fig. 3-10) confirmed the presence of 6 *m/z* 535 peaks and 6 MH^+ ions consistent with C_{27}, C_{28}, and C_{29} mono- or di-unsaturated sterols esterified to pyrophaeophorbide *a*. Each peak has the typical mass spectrum (figs. 3-11 to 3-16) consisting of MH^+ and fragment ion at *m/z* 535 indicating the pyrophaeophorbide *a* nucleus. The protonated molecular ions and corresponding sterols are given in Table 3-2.

Peak*	SCE MH^+	Esterifying Sterol
a, b	901	C_{27} 2 double bonds
d	903	C_{27} 1 double bond
c	915	C_{28} 2 double bonds
e, f	929	C_{29} 2 double bonds

Table 3-2. *SCE MH^+ and corresponding sterol (*see fig. 3-10).*

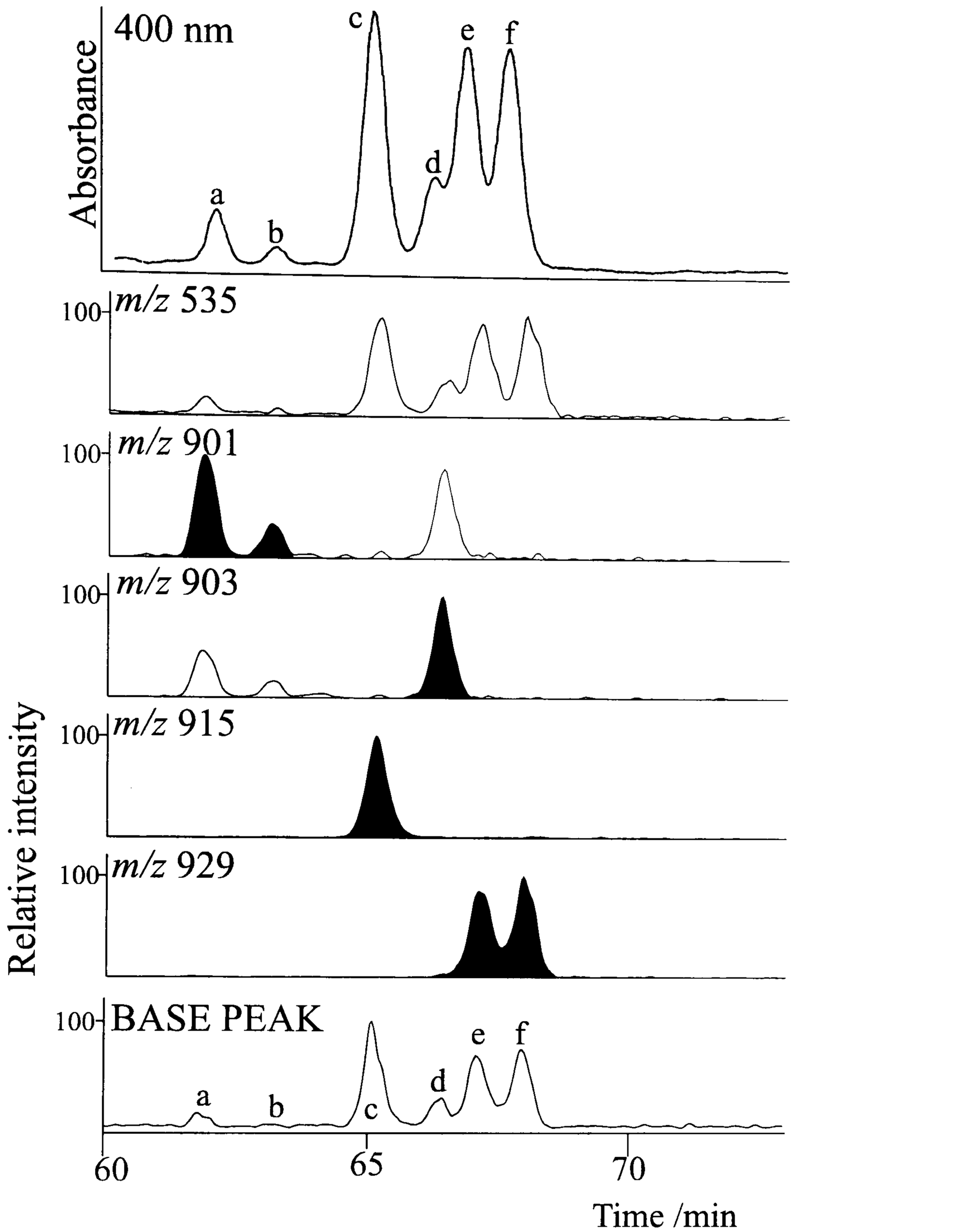


Figure 3-10. *Faecal pellet SCE region mass chromatograms and HPLC chromatogram (410 nm), shaded peaks are SCE MH^+.*

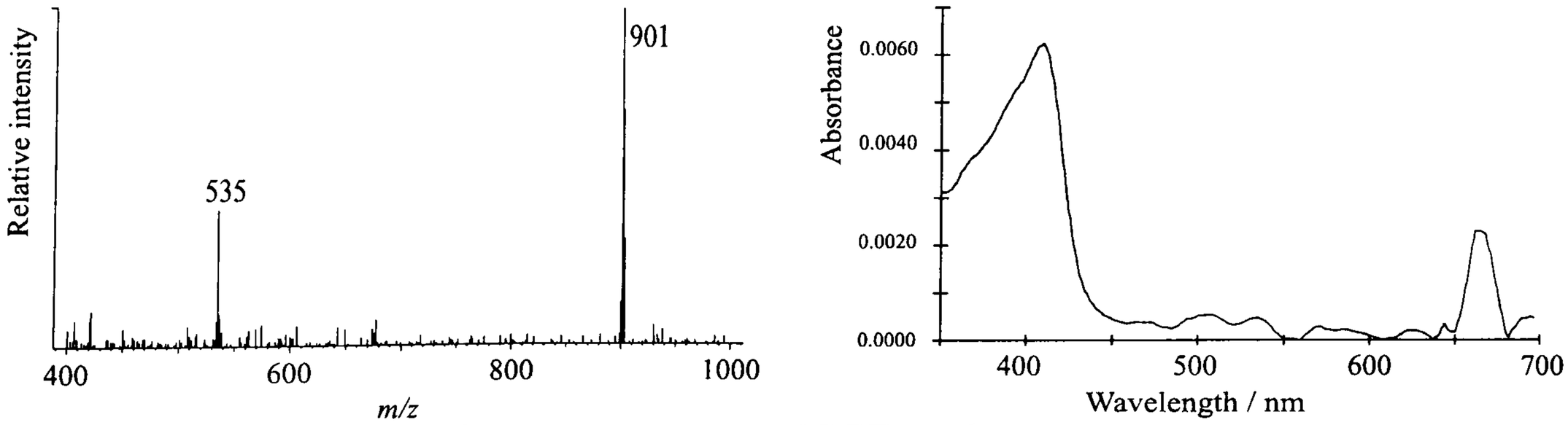


Figure 3-11. *Mass and electronic spectra of SCE peak a.*

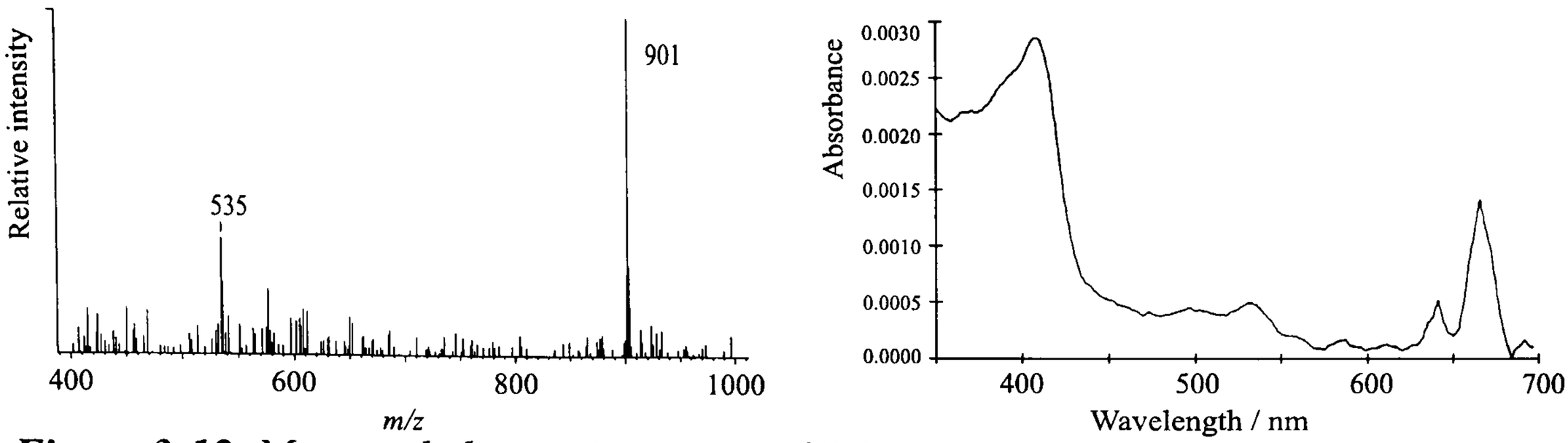


Figure 3-12. *Mass and electronic spectra of SCE peak b.*

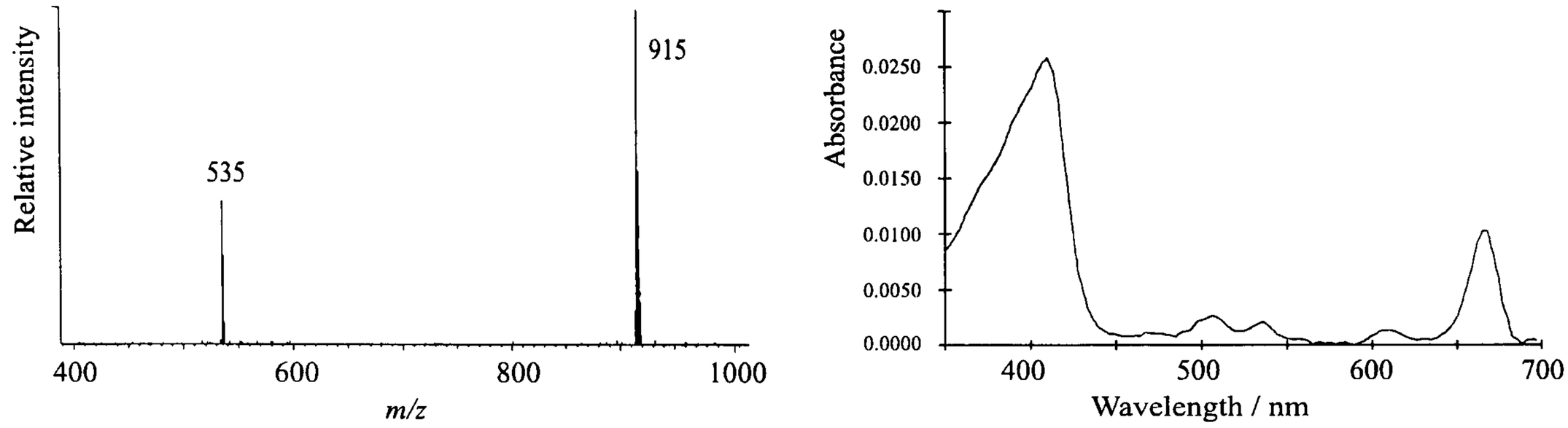


Figure 3-13. *Mass and electronic spectra of SCE peak c.*

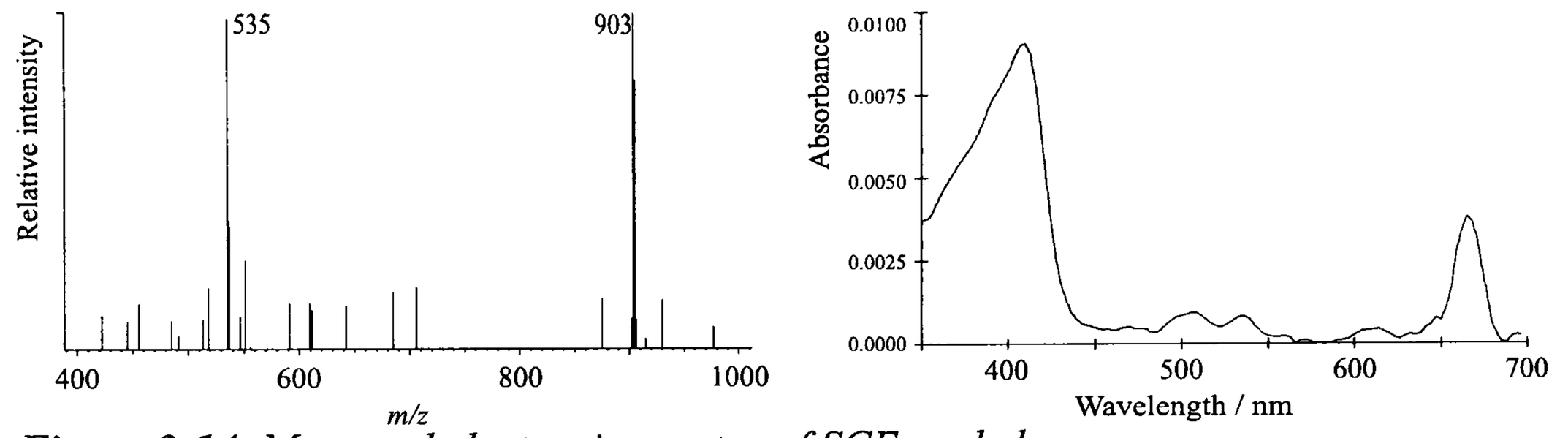


Figure 3-14. *Mass and electronic spectra of SCE peak d.*

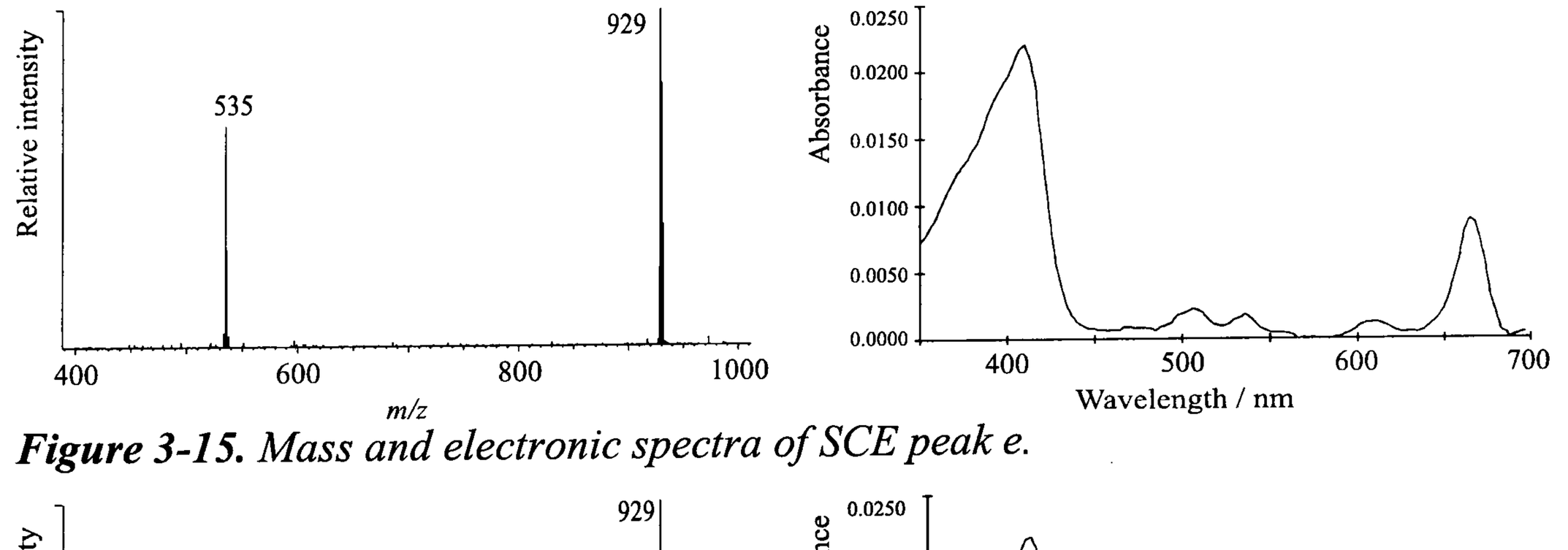


Figure 3-15. *Mass and electronic spectra of SCE peak e.*

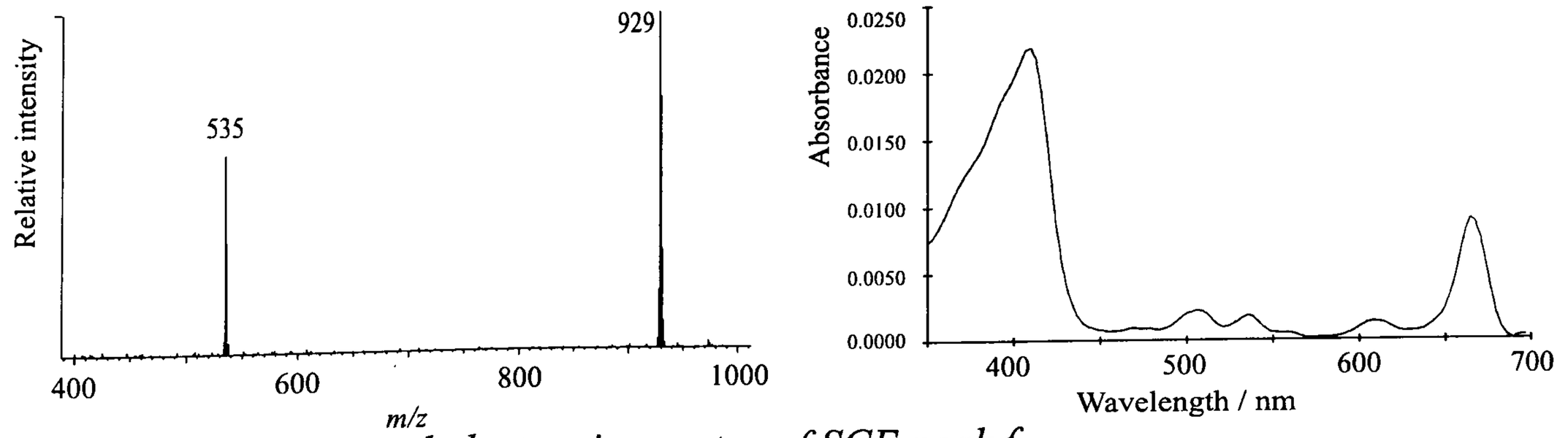


Figure 3-16. *Mass and electronic spectra of SCE peak f.*

The SCEs were found to comprise *ca.* 55% of the total pellet chlorin components, which is unusually high compared to the other feeding experiments which produced SCEs.

3.3.4 Free Sterols

3.3.4.1. Pre-Starved Copepods

The starved animals contained only three sterols, the distribution being dominated by cholest-5-en-3β-ol (**A1**) with lower amounts of cholesta-5,24-dien-3β-ol (**A2**) and cholesta-5,22-dien-3β-ol (**A3**), similar to that found in the large scale prasinophyte experiment (Chapter 2).

3.3.4.2. Algal Culture

The algal culture free sterol distribution contained four major (peaks 1, 3-5; fig. 3-17) and one minor component (peak 2).

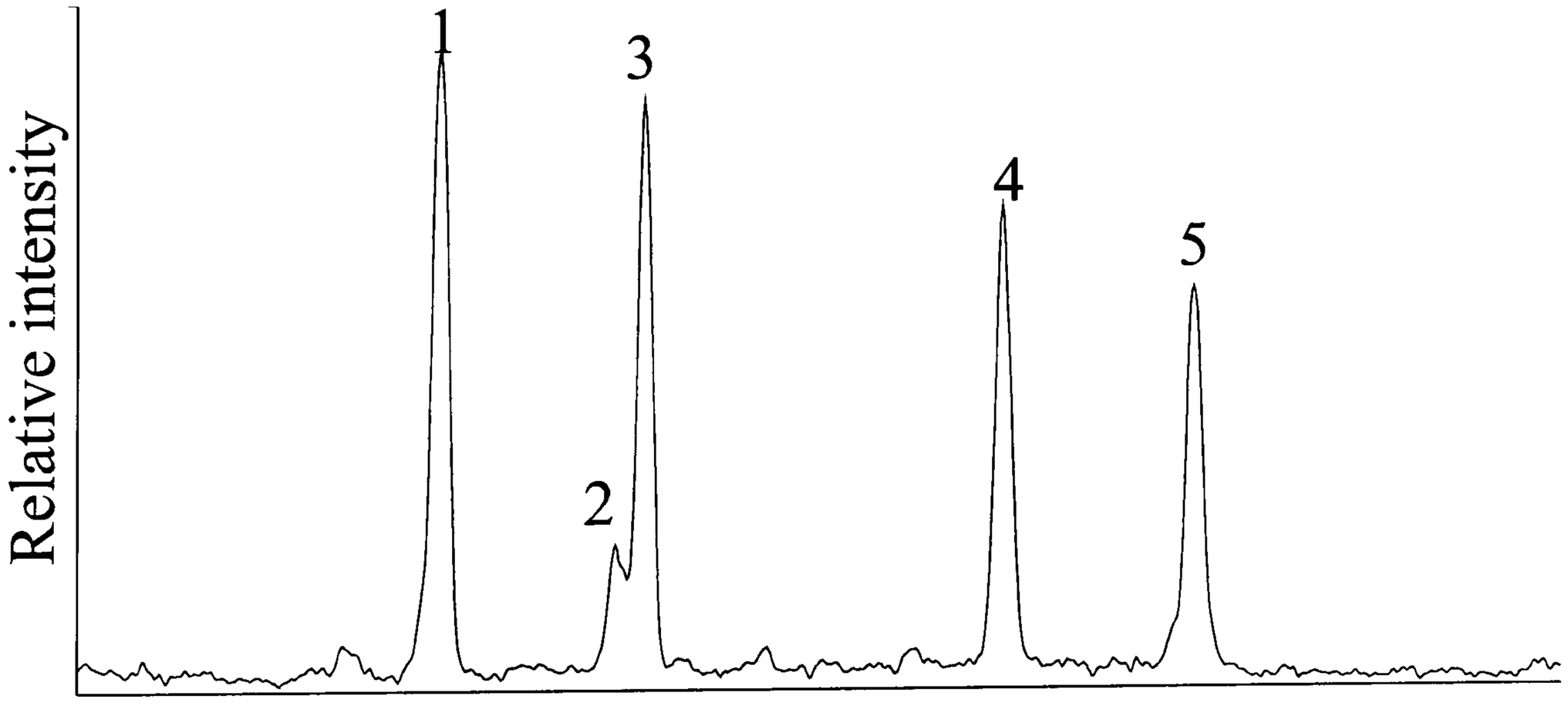


Figure 3-17. *Partial RIC trace of P. carterae free sterols (as TMSi ethers)*

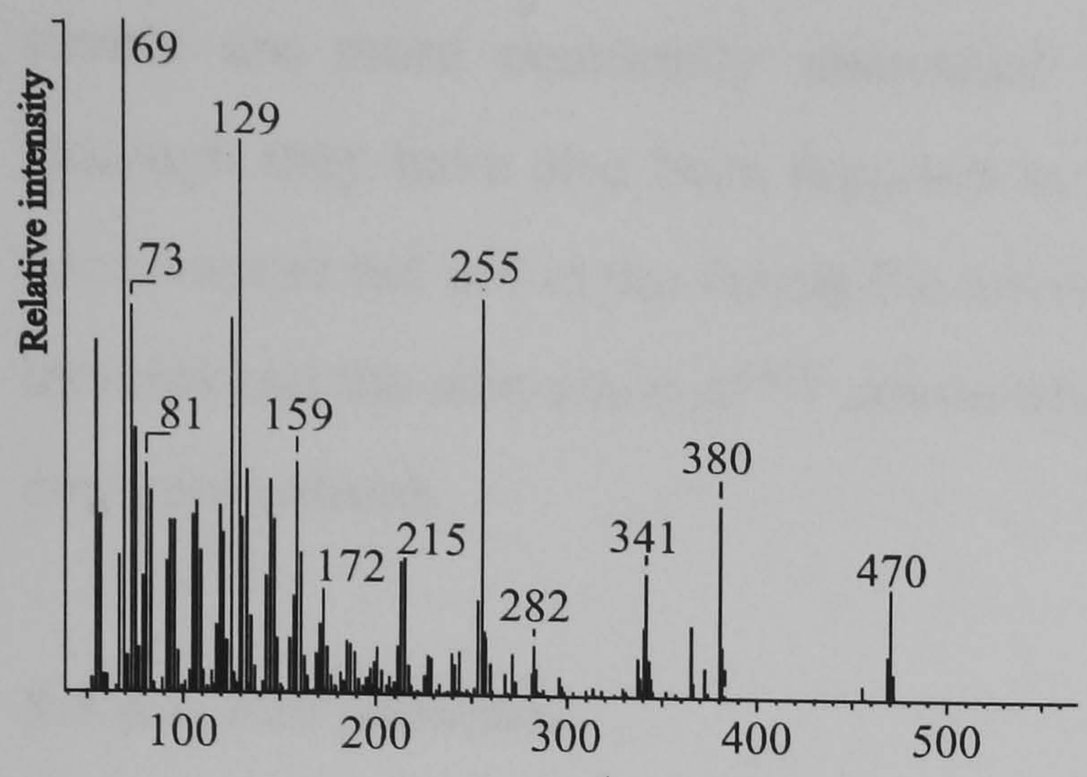
Peak 1 is assigned as 24-methylcholesta-5,22-dien-3β-ol (**A6**; fig. 3-18a) and peak 3 as 23,24-dimethylcholesta-5,22-dien-3β-ol (**A10**; fig. 3-18c). The mass spectrum of peak 4 (fig. 3-18d), although similar to that of 24-ethylcholesta-5,22-dien-3β-ol, can not be that component as it would elute significantly closer to peak 3 (*cf.* peaks 7 and 8, fig. 5-24; Chapter 5). Assignment of this component as 24-ethylcholesta-5,24(28)-dien-3β-ol is ruled out due to the absence of an ion at *m/z* 386 (Goad, 1991). As this component elutes close to 23,24-dimethylcholesta-5,22-dien-3β-ol (peak 3) it is probable that it also has a 23,24-dimethyl substituted side chain rather than a C-24 ethyl side chain

component. The ion at *m/z* 372 suggests a $\Delta^{23(28)}$ bond in the side chain analogous to that observed at *m/z* 386 in peak 5 (see below); therefore this component is tentatively assigned as 23,24-dimethylcholesta-5,23(28)-dien-3β-ol (**A12**). The fourth major component, peak 5 (fig. 3-18e), appears to be a 4-methyl component with 23,24-dimethyl alkylated side chain (from the presence of an ion at *m/z* 69); the spectrum does not, however, match that of 4α,23,24-trimethylcholesta-5,22-dien-3β-ol (**B10**; Volkman, personal communication). The ion at *m/z* 386, which is normally associated with cleavage of the C-22/C-23 bond in a side chain with a $\Delta^{24(28)}$, in this case would have to be $\Delta^{23(28)}$ due to the presence of the 4-methyl group. Therefore this component is tentatively assigned as of 4α,23,24-trimethylcholesta-5,23(28)-dien-3β-ol (**D12**). The minor component peak 2 again appears to have the 23,24-dimethyl Δ^{22} side chain (*m/z* 69) with one double bond in the ring system at C-5 indicated by the ions at *m/z* 129 and 255. Recently, 24-ethylcholesta-5,22,25-trien-3β-ol has been reported to elute just prior to 24-ethylcholesta-5,22,25-trien-3β-ol (Volkman, personal communication) so this component is tentatively assigned as 23,24-dimethylcholesta-5,22,25-trien-3β-ol (**A13**). All culture sterol assignments are summarised in Table 3-3.

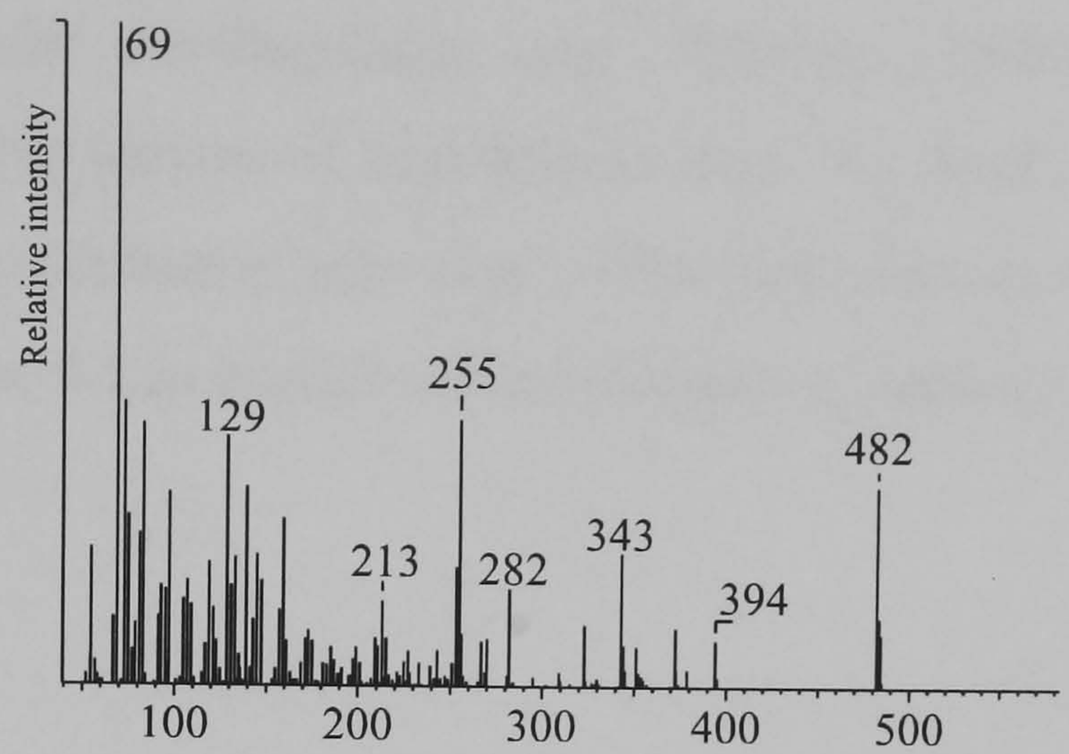
Peak*	Assignment	Abbreviation	Structure
1	24-methlycholesta-5,22-dien-3β-ol	$C_{28}\ \Delta^{5,22}$	A6
2	23,24-dimethylcholesta-5,22,25-trien-3β-ol	23,24-Me $C_{29}\ \Delta^{5,22,25}$	A13
3	23,24-dimethylcholesta-5,22-dien-3β-ol	23,24-Me $C_{29}\ \Delta^{5,22}$	A10
4	23,24-dimethyl cholesta-5,23(28)-dien-3β-ol	23,24-Me $C_{29}\ \Delta^{5,23(28)}$	A12
5	4α,23,24-trimethylcholesta-5,23(28)-dien-3β-ol	4α-Me $C_{30}\ \Delta^{5,23(28)}$	D12

Table 3-3. *P. carterae sterol assignments and structures (*fig. 3-17).*

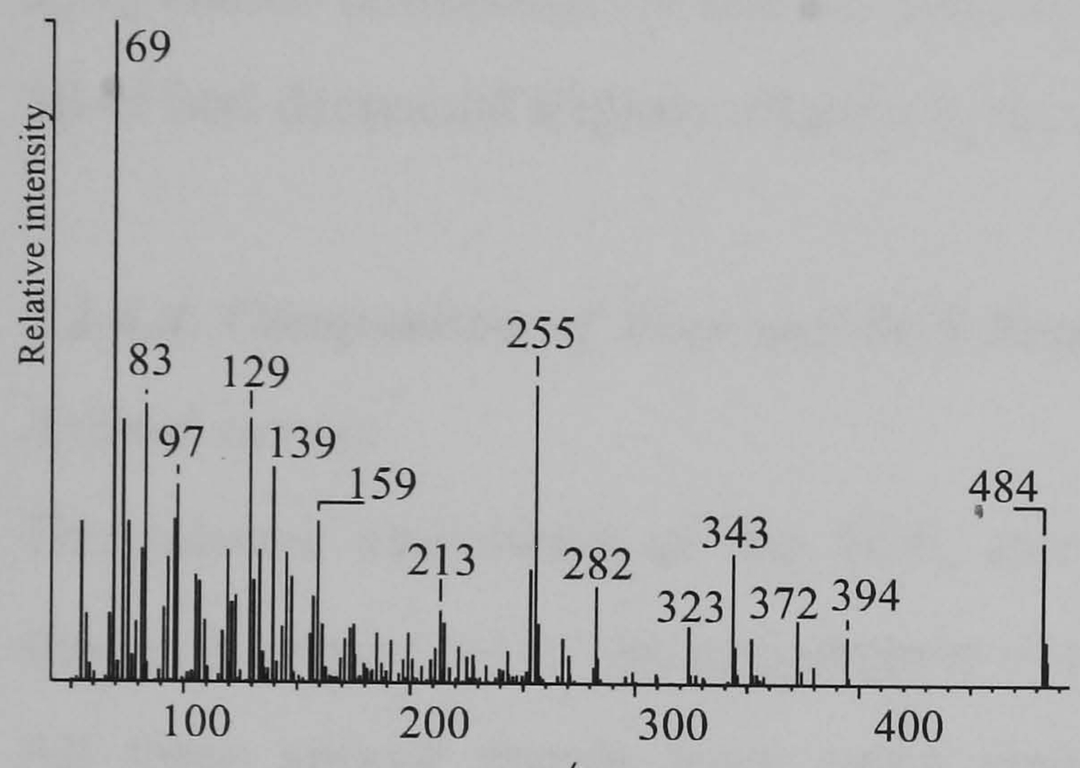
(a) Peak 1 C_{28} $\Delta^{5,22}$



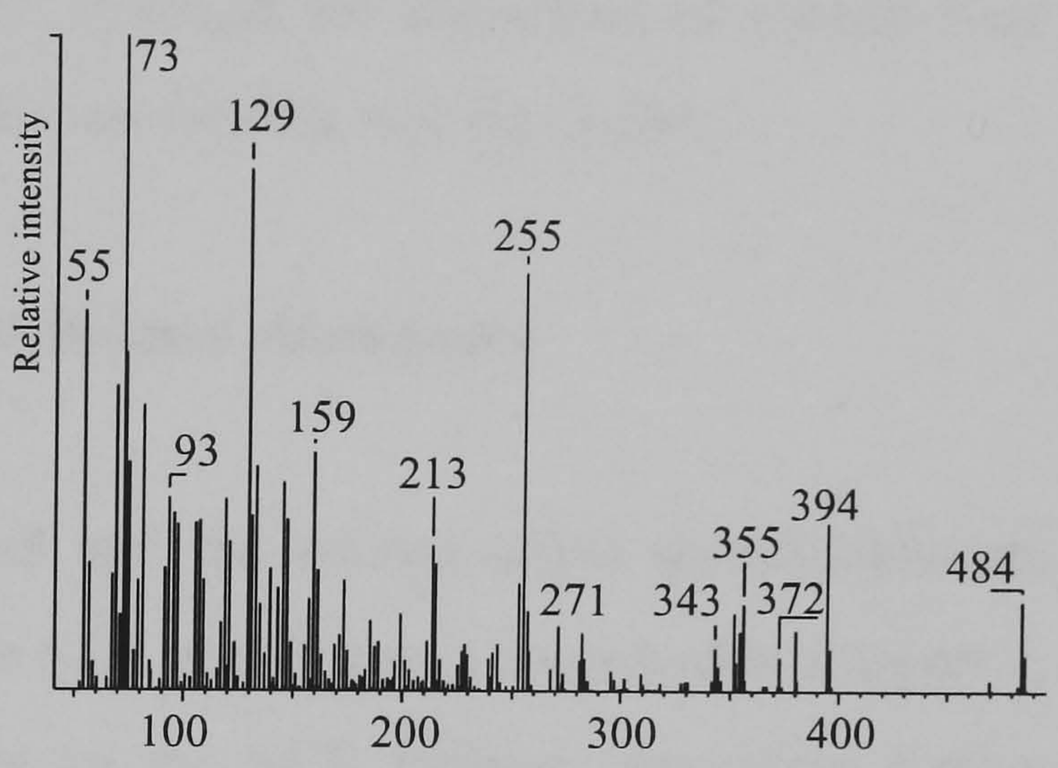
(b) Peak 2 23,24-diMe C_{29} $\Delta^{5,22,25}$



(c)Peak 3. 23,24-diMe C_{29} $\Delta^{5,22}$



(d) Peak 4. 23,24-diMe C_{29} $\Delta^{5,23(28)}$



(e) Peak 5. 4α-Me C_{30} $\Delta^{5,23(28)}$

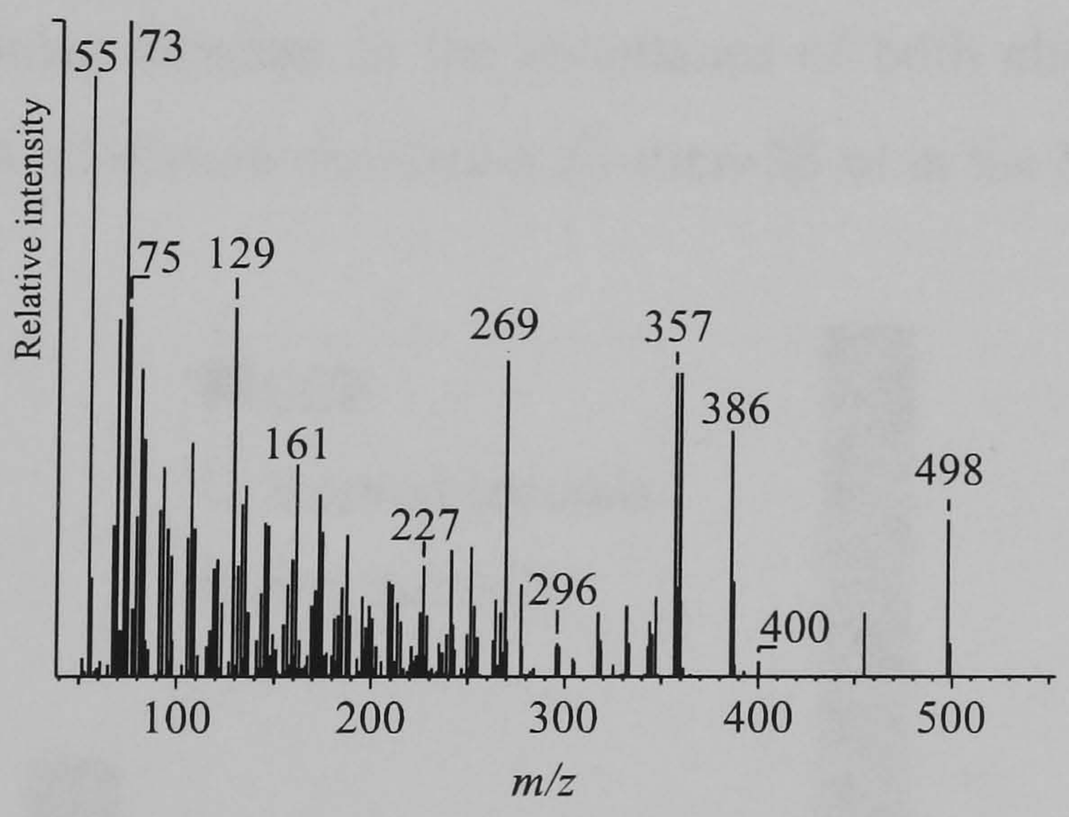


Figure 3-18. *Mass spectra of P. carterae sterols (as TMSi ethers).*

The presence of peaks 1 and 3 is consistent with the observations of earlier workers (Conte *et al.* 1994 and references therein) who found these two sterols along with 24-ethylcholesta-5,22-dien-3β-ol as the only sterols in this organism. The presence of the minor C_{29} trienol is unexpected as this structure was not observed to be among the major components of any of the sterol profiles of 21 different haptophyte species summarised

by Conte *et al.* (1994). The proposed 4-methyl sterol is also unexpected as 4-methyl sterols are more commonly associated with dinoflagellates (e.g. Volkman, 1986) although they have also been reported in five species of haptophytes from the family Pavlovaceae but not in the family Pleurochrysidaceae (Conte *et al.*, 1994 and references therein) and the side-chain $\Delta^{23(28)}$ unsaturation is also highly unusual (Volkman, personal communication).

3.3.4.3. Fed Animals

The distribution was similar to that of the starved copepods, again showing three components dominated by cholest-5-en-3β-ol, although the abundance of cholest-5-en-3β-ol had decreased slightly relative to the starved animals (see fig. 3-19).

3.3.4.4. Comparison of Free and SCE Sterol Relative Abundance

Animal sterols

The relative abundance of the SCE, starved and fed animal sterol distributions are shown in Figure 3-19. The assignments of the SCE sterols are as described in Chapter 2. All three animal sterols were again present in the SCE fraction, providing further evidence that SCEs are qualitative indicators of herbivore sterol distributions. There was, however, a significant increase in the abundance of both cholest-5-en-3β-ol and cholesta-5,24-dien-3β-ol relative to cholesta-5,22-dien-3β-ol in the SCE fraction.

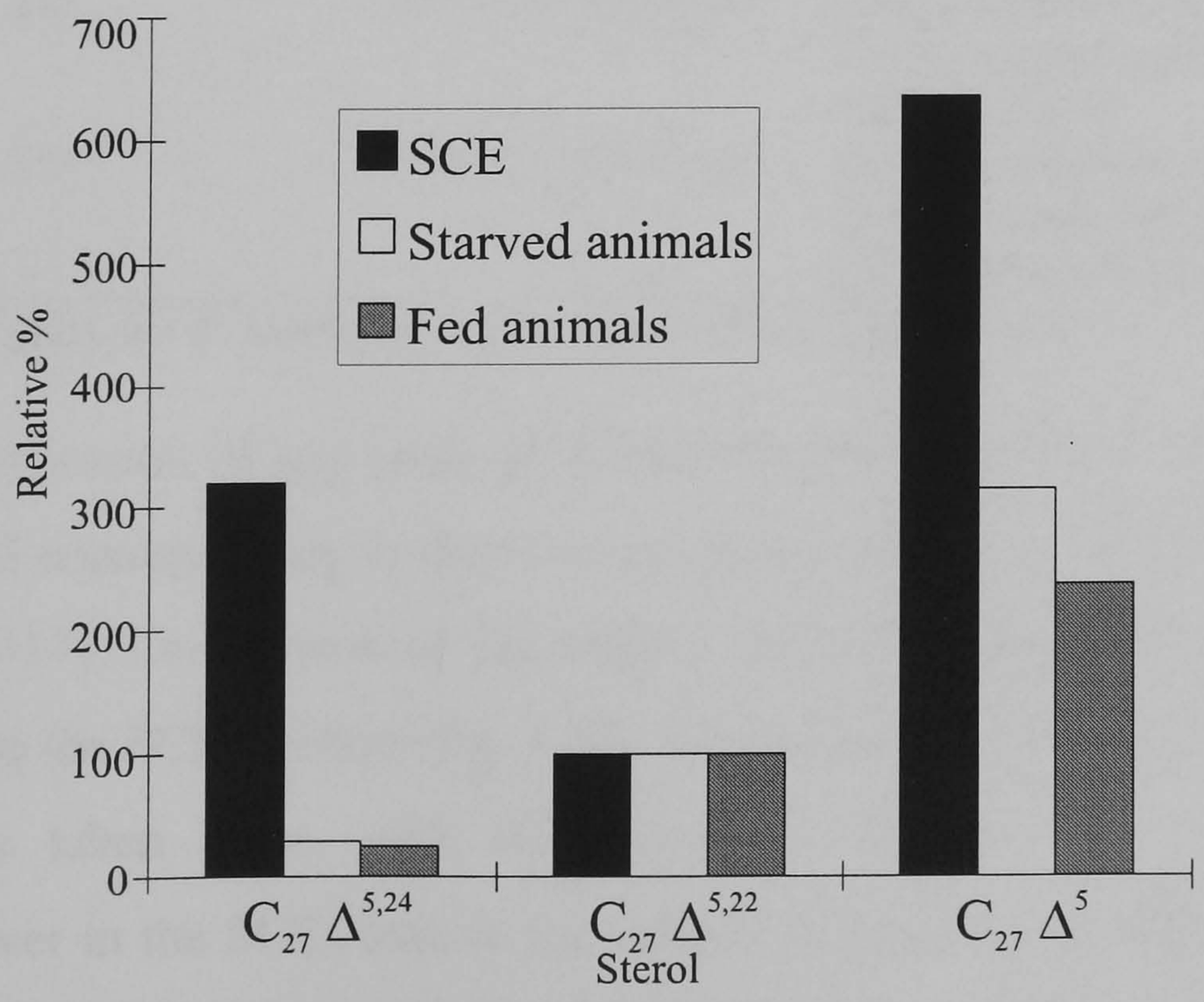


Figure 3-19. *Relative % of animal sterols in SCEs, starved and fed copepods.*

Culture sterols

Comparison of the culture sterol and SCE sterol distribution was more complicated. Five sterols were found in the culture but only three SCEs with MH^+ values corresponding to those of the culture sterols were detected in the faecal pellet extract. There was only one sterol available corresponding to the SCE with MH^+ at *m/z* 915 so SCE peak c (fig. 3-10, Table 3-4) was assigned as the 24-methylcholesta-5,22-dien-3β-ol ester. As no standards were available of the two C_{29} diunsaturated steryl esters assignment of the SCEs is tentative based on the observation of the relative retention times of cholest-5-en-3β-ol and 23,24-dimethylcholest-5,22-dien-3β-ol in the SCEs produced during grazing on the dinoflagellate *Prorocentrum micans* (see Chapter 5) in which this C_{29} SCE elutes immediately after the cholest-5-en-3β-ol SCE. Hence peak e is assigned as the 23,24-dimethylcholesta-5,22-dien-3β-yl ester and peak f as the 23,24-dimethylcholesta-5,23(28)-dien-3β-yl ester.

Peak*	MH^+	other ions	λ_{max} /nm	Assignment	Structure
a	901	535	410,503,536,611,665	pyrophaeophorbide *a* cholesta-5,24-dien-3β-ol	**X-A2**
b	901	535	410,503,536,611,665	pyrophaeophorbide *a* cholesta-5,22-dien-3β-ol	**X-A3**
c	915	535	410,503,536,611,665	pyrophaeophorbide a 24-methylcholesta-5,22-dien-3β-ol	**X-A6**
d	903	535	410,503,536,611,665	pyrophaeophorbide *a* cholest-5-en-3β-ol	**X-A1**
e	929	535	410,503,536,611,665	pyrophaeophorbide *a* 23,24-dimethylcholesta-5,22-dien-3β-ol	**X-A10**
f	929	535	410,503,536,611,665	pyrophaeophorbide *a* 23,24-dimethylcholesta-5,23(28)-dien-3β-ol	**X-A12**

Table 3-4. *SCE data for P. carterae experiment (* see fig. 3-10).*

There was no indication of any other SCE components in the mass chromatograms of *m/z* 927 and 943 corresponding to the two remaining culture sterols (minor peak 2 and peak 5 in fig. 3-17). Comparison of the relative abundance of the three culture sterols incorporated into the SCE fraction (fig. 3-20) shows that significant modification of the distribution has taken place, with the abundance of the two C_{29} sterols being significantly lower in the SCEs than in the culture free sterols. Overall, the SCE sterol distribution was dominated by sterols originating from the haptophyte which comprised *ca.* 85% of the total, with the remainder originating from the copepod.

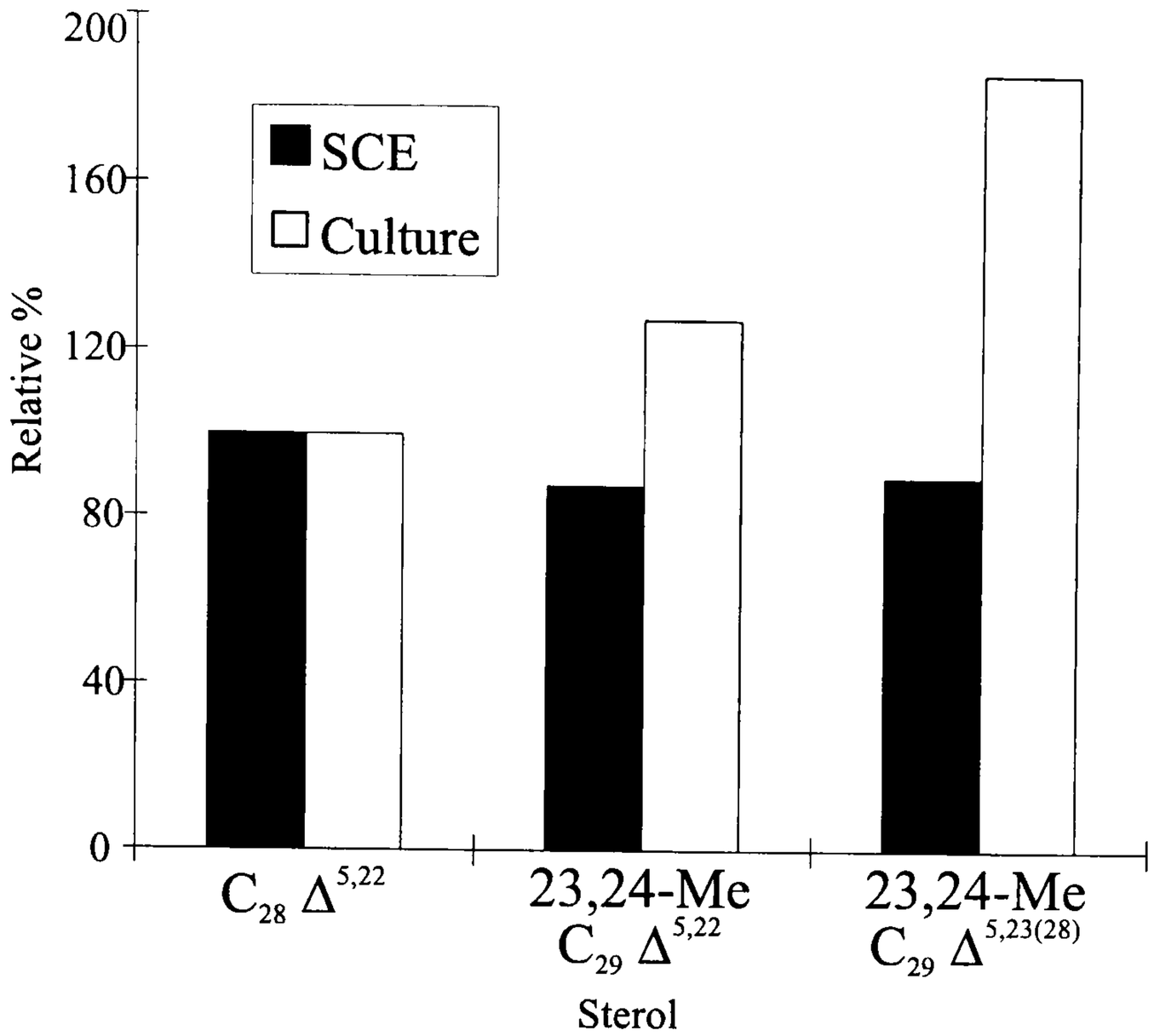


Figure 3-20. *Relative % of algal sterols in SCEs and culture.*

3.4. DISCUSSION

3.4.1. Pigments

Production of SCEs was not seen in the small scale experiment with the non-coccoid haptophyte *I. galbana* although they were observed in similar scale experiments with different algae (chlorophyte, Chapter 2; diatom, Chapter 4). The copepod *C. helgolandicus* would seem to graze well on this organism, a soft bodied flagellate, resulting in high assimilation efficiency of the algal material and leading to low pellet production. This behaviour has been observed previously (Harris, personal communication), so it was decided that to repeat this experiment on a sufficiently large scale to produce enough material for analysis would not be possible given the facilities at hand; hence it is not possible to say whether SCEs would be seen with other non-coccoid haptophytes, although it would seem likely.

When feeding on the coccolithophorid *C. pelagicus*, again no SCEs were observed although this experiment was performed on a larger scale (x 3) than that with *I. galbana* and pellet production was very high. However, the organic carbon and hence pigment

content of this alga was very low so the pigment content of the pellets was also very low, presumably due at least in part to high assimilation of the available organic carbon. The use of remote sensing has shown that coccolithophores do not produce a strong pigment signature (Harris, personal communication). Of the three species used in the present study *C. pelagicus* has the most similar characteristics to *E. huxleyi*, with these two coccolithophorids being the two most common bloom forming species in the open ocean, suggesting therefore that SCE production from haptophytes in the open ocean would be low. Although the numbers of *C. pelagicus* in a bloom are generally lower than those of *E. huxleyi* it can account for a large proportion of the biomass as it is a much larger cell than *E. huxleyi* (Harris personal communication). *E. huxleyi* was not used in the present study as some studies have shown poor pellet production from *Calanus* feeding on this species (Harris, personal communication). Again repetition of this experiment on a scale large enough to produce sufficient material to expect a positive SCE result would have been impractical given the facilities needed to grow sufficient culture and to perform the feeding experiment.

Unlike *I. galbana* and *C. pelagicus*, SCEs were observed with the coccolithophorid *P. carterae* (a coastal species), at a scale similar to that of the *C. pelagicus* experiment. *P. carterae* is intermediate in size to the other two species used and with a significantly higher pigment content than *C. pelagicus* and higher pellet production rate than with *I. galbana*. It would seem therefore that efficiency of SCE formation is variable in haptophytes and is not only dependent on chl content as expected but also on cell size. The presence of a coccosphere also seems to play a role; *C. pelagicus* is more heavily armoured than *P. carterae*. It has previously been observed that the activity of digestive enzymes in the copepod gut is dependent on pH, with an optimal activity range being lower than the normal pH of seawater i.e. pH 5-7.5 for most crustaceans (Pond *et al.*, 1995 and references therein). However, if dissolution of the ingested calcite liths is extensive, this may result in a gradual increase in pH, thus reducing the activity of the enzymes and consequently having an effect on the extent of pigment degradation as enzymatic degradation of chl *a* is facilitated by the presence of acid. This phenomenon is thought to be most apparent when copepods have undergone a period of starvation

which leads to an increase in the pH of the foregut, the site of acid secretion, resulting in initial dissolution of the coccoliths until the pH rises above that of normal seawater.

3.4.2. Sterols

In the *P. carterae* experiment all of the animal sterols were present in the SCE fraction; however, the SCE distribution demonstrated significant alteration prior to esterification, attributed to additional production of cholesta-5,24-dien-3β-ol and cholest-5-en-3β-ol from the ingested phytosterols (e.g. Goad, 1978, 1981; see also Chapter 2). As the third animal sterol cholesta-5,22-dien-3β-ol has not been implicated in the C-24 dealkylation pathway it would seem to be a side product. If so the increase in abundance of these two sterols as esters originates from their production in excess of the nutritional requirements of the copepod as suggested by Prahl *et al.* (1984) with respect to the high abundance of free cholesterol in copepod faecal pellets.

There were two main differences in the culture sterols and SCE sterols. Firstly, and uniquely during this work, two of the algal sterols, a C_{29}-trienol and a C_{30} dienol (incorporating a 4-methyl substituent) were not observed in the SCE fraction. It should be noted, however, that to date no examples of triunsaturated sterols have been reported in hydrolysed sedimentary and water column particulate SCE fractions (Eckardt *et al.*, 1991b, 1992; King and Repeta, 1991, 1994; Chillier and Gülaçar, 1995; King and Wakeham, 1996; Pearce *et al.*, 1998; Laureillard *et al.*, 1997) and there is only one mention of a possible C_{30} diunsaturated component which was not rigorously assigned as a sterol (Eckardt *et al.*, 1992). This could be seen to indicate either that the overall abundance of these two types of components is low in the natural environment or that esterification of sterols such as these is hindered in some way. However, the C_{29} trienol was present in low abundance relative to the other sterols (peak 2; fig. 3-17), which might make it more difficult to observe as an SCE. On the other hand, the SCEs were the most abundant components in the pellets so it is expected that the appropriate *m/z* 927 SCE would have been apparent using mass chromatography. It is possible, however, that this trienol was not observed in the esterified form as it was assimilated by the copepod with particularly high efficiency. Prahl *et al.* (1984), during a study with

Calanus grazing on the chlorophyte *Dunaliella primolecta*, found two sterol trienes (in that case $\Delta^{5,7,22}$) in high abundance (i.e. ≥ 8%), but, both were absent from the faecal pellets produced.

In the case of the C_{30} 4-methyl sterol, this abundant component would have been expected to be observed since 4-methyl sterol SCEs were formed in other experiments (Chapter 5). Although there is discrimination against them in forming SCEs (see Chapter 5), the effect is not sufficiently great for the component not to be observed as an SCE. The absence of this component is difficult to explain at present but may be related to the observations of Harvey *et al.* (1987) that a number of ring unsaturated 4α-methyl sterols which were present in the dinoflagellate *Scrippsiella trochoidea* were present in pellets produced during grazing by the copepod *C. helgolandicus* in significantly lower abundance than in the substrate, suggesting high assimilation of such components. This factor and the apparent discrimination against incorporation of 4-methyl sterols into SCEs (see Chapter 5) could explain, at least in part, the absence of this component in the pellet SCE fraction.

The second difference is the relative abundance of the three diunsaturated sterols which were incorporated as SCEs. The abundance of the two C_{29} sterols was lower in the SCE fraction than in the culture (fig. 3-22). This observation would seem to suggest that the C_{29} sterols were more readily assimilated by the copepod for conversion to cholest-5-en-3β-ol. This apparently contradicts the observations of Bradshaw *et al.* (1989) who found that C-24 dealkylation proceeds more efficiently with 24-methyl sterols than 24-ethyl sterols in a number of marine invertebrates including crustaceans. However, in this case the C_{29} sterols both had C-23/24 dimethyl substitution, whereas the C_{28} sterol had only C-24 substitution. Perhaps the additional carbon at C-23 enhances assimilation. Unfortunately insufficient pellet material was available after the SCE studies to isolate and determine the distribution of the free sterols to investigate this effect.

3.5. SUMMARY

SCE formation from haptophytes has been confirmed for the first time when a copepod grazed on the haptophyte *P. carterae* (coccolithophorid). However, two other experiments with different haptophytes (the non-coccoid *I. galbana* and the coccolithophorid *C. pelagicus*) failed to show SCE production, possibly due to amounts being below detection limits. This species dependence suggests that in the natural environment, a haptophyte input to sediments deduced from SCE sterol distributions could be underestimated, depending on the cell dimensions and the presence and extent of calcification. The observations with *C. pelagicus* could be of particular importance in open ocean settings when coccolithophorids, especially *E. huxleyi* (see above) are major constituents of the phytoplankton population.

Chapter 4

FEEDING EXPERIMENTS WITH A DIATOM

4.1. INTRODUCTION

4.1.1. Background

It is now well established that SCEs are significant components of the chlorin fractions in bottom sediments (e.g. *ca*. 40% Black Sea bottom sediment, King and Repeta, 1991). Their significant or high abundance suggests indirectly the possibility that they could be more stable relative to other more frequently monitored chlorophyll transformation products e.g. phaeophytin *a* (**VIII**) and phaeophorbide *a* (**VII**); if so, this could have important consequences for the use of these pigments as both grazing indicators (*c.f.* Goericke *et al*., 1999) and indicators of phytoplankton palaeocommunity structure (King and Repeta, 1994; Pearce *et al*., 1998). In order to investigate the stability of the distribution of SCE-esterified sterols and their possible greater resistance to degradation relative to other chlorins it was necessary to perform a large scale feeding experiment and monitor the changes in the faecal pellet pigment signatures during ageing (see also Chapter 5).

4.1.2. Present Study

The first laboratory study to demonstrate SCE production (Harradine *et al*., 1996b) involved *Calanus helgolandicus* grazing on a diatom (*Thalassiosira weissflogii*). It was decided that the first stage of the present work would be to repeat this preliminary experiment on a similar small scale to confirm the earlier observations and also to extend it by analysing the contribution of animal sterols to the SCEs in this case. This experiment demonstrated a high pellet production rate by *C. helgolandicus* when feeding on *T. weiss*, so the latter was chosen as a suitable substrate for a larger scale study involving examination of the pellet pigment signatures at different stages of ageing and investigating changes in both the distribution and abundance of SCE in faecal pellets as they underwent ageing.

The small scale experiment pigment extracts were methylated prior to analysis by HPLC-MS, leading to the production of an unexpectedly high proportion of methylated

artefacts. This practice was therefore abandoned for all subsequent experiments (e.g. Chapters 2 and 3). This and the use of an ODS2 HPLC column rather than an ODS3 column in subsequent experiments did mean, however, that the HPLC results for the small scale experiment showed poorer resolution, so only a brief discussion is given of the SCE data from this experiment. The main discussion is based on the results of the second, large scale experiment incorporating the ageing studies, an outline of which is given in Figure 4-1. It should be noted, however, that the electronic data from the original analysis of this experiment were lost due to a hard disk failure.

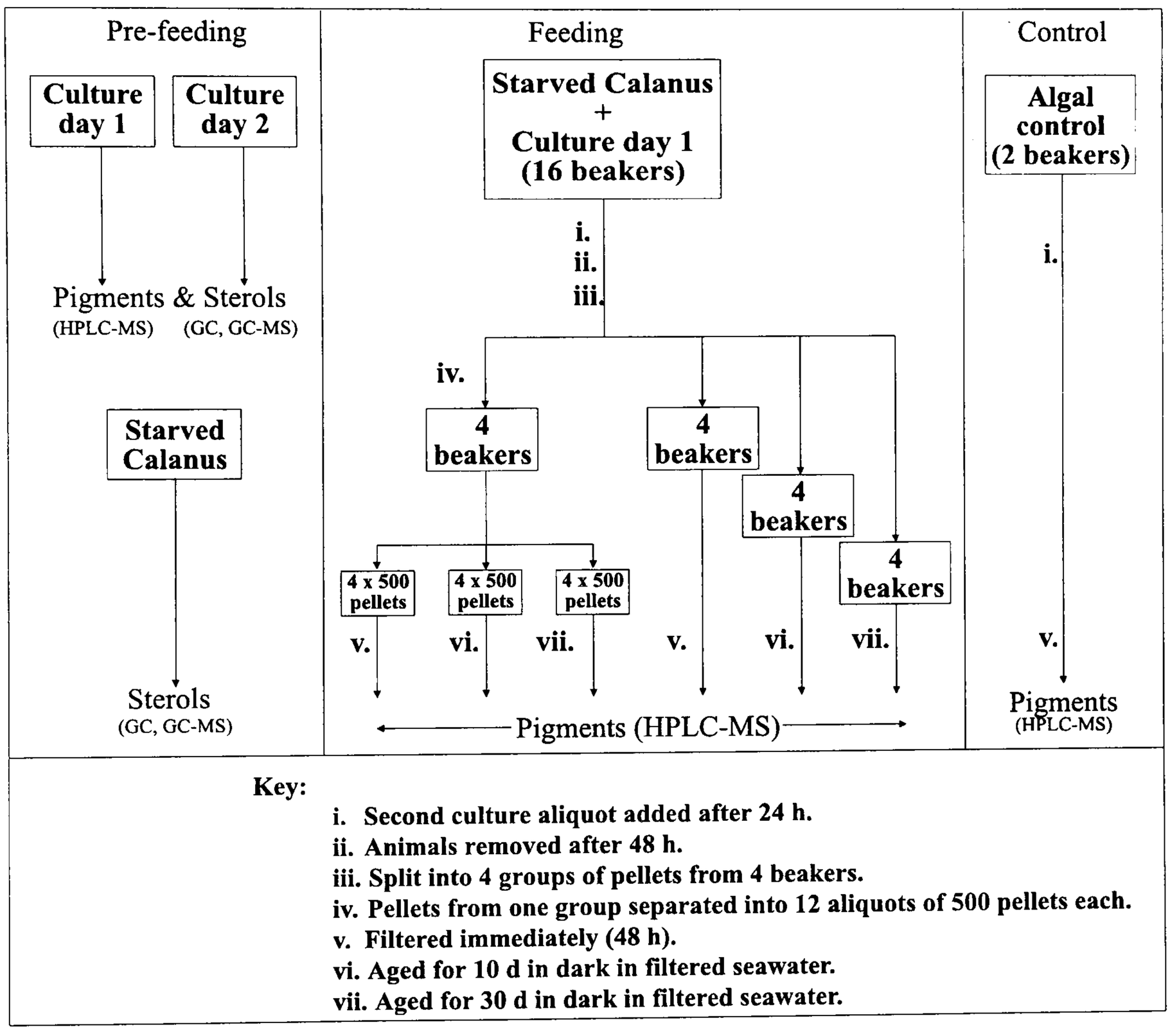


Figure 4-1. *Outline of samples and analysis protocols for large scale T. weiss feeding experiment.*

4.1.3. The Bacillariophyta (Diatoms)

The Bacillariophyta or diatoms are the best known of the unicellular phytoplanktonic algae and occur ubiquitously in all types of marine and lacustrine environments. There are 210 known genera and at least 5000 living species with about twice as many found in the fossil record (Jeffrey and Vesk, 1997).They are found in all phytoplankton size

classes but are generally between 5 and 200μm, with a few as large as 2mm. Their most characteristic feature is a box-like wall or frustule made of silica which surrounds the non-flagellated cell. Diatoms can occur as symbionts in some larger foraminifera, clams and corals, although dinoflagellates are more commonly associated with this role. They are often bloom-forming species with a similar species succession occurring in most situations. Small rapidly growing, chain-forming species appear first followed by larger centric diatoms. When the silica for wall construction is depleted the diatoms are replaced by flagellates. They are divided into two orders based on the symmetry of their structure with Centrales (or centric diatoms) having radial symmetry around a point or points and Pennales (or pennate diatoms) being symmetrical around a line. *Thalassiosira weissflogii* is a centric diatom, 12-20 μm in diameter, which although not commonly found in the open ocean, is considered to be a good model diatom species (Harris, personal communication).

Diatoms contain a pigment distribution similar to that of the haptophytes (Chapter 3), with the main components being chl *a* (**I**), chl c_1 and c_2 (**III, IV**), fucoxanthin (**XXVI**), diadinoxanthin (**XXXVI**), diatoxanthin (**XXXVII**) and β,β-carotene (**XXXV**) (Jeffrey and Vesk, 1997). In a small number of species chl c_3 replaces chl c_1 (Stauber and Jeffrey, 1988).

4.2. RESULTS: PRELIMINARY EXPERIMENT

4.2.1. Pigments

A few difficulties were encountered during the small scale experiment. A high proportion of oxygenated and methylated artefacts were observed in the pigment distributions with , with no evidence of chl *a* and very little unaltered phaeophytin *a* being present. The artefacts are thought to have been produced largely due to the use of diazomethane for methylation of the samples prior to analysis of HPLC (*cf.* Eckardt *et al.*, 1991b; Harradine *et al.*, 1996b) specifically as a result of the addition of the diazomethane in ether solution which appeared to have contained peroxide radicals. However, SCEs were still clearly observed in the extracts of pellets plus remaining alga

and faecal pellets separated from remaining alga aged for 35 d. A total of 8 components were identified in the SCE region by mass chromatography (fig. 4-2), each displaying an electronic spectrum similar to that of pyrophaeophorbide *a*.

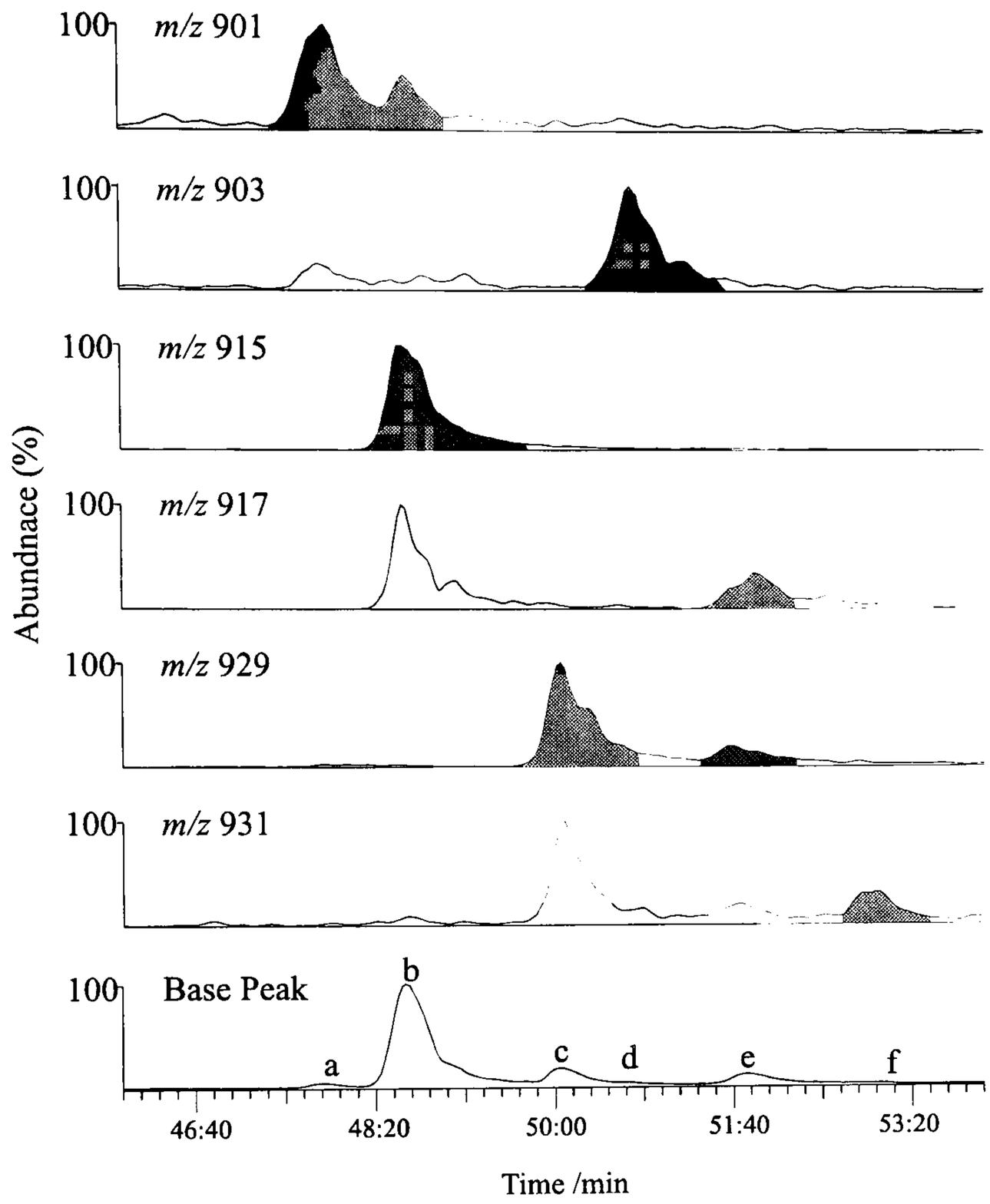


Figure 4-2. *Mass chromatograms of SCE MH^+ from aged pellets.*

The MH^+ ions of the SCEs and their corresponding esterified sterols are given in Table 4-1. Mass spectra of the individual peaks all showed the expected fragment ion at *m/z* 535.

Peak	SCE MH^+	Esterifying Sterol
a	901	C_{27} 2 double bonds
b	901	C_{27} 2 double bonds
d	903	C_{27} 1 double bond
b	915	C_{28} 2 double bonds
e	917	C_{28} 1 double bond
c	929	C_{29} 2 double bonds
e	929	C_{29} 2 double bonds
f	931	C_{29} 1 double bond

Table 4-1. *SCE MH^+ and corresponding sterol.*

As before (*cf.* Chapter 2), there was some co-elution among the SCEs so individual components were again quantified from their MH^+ peak areas in the mass chromatograms (fig. 4-3).

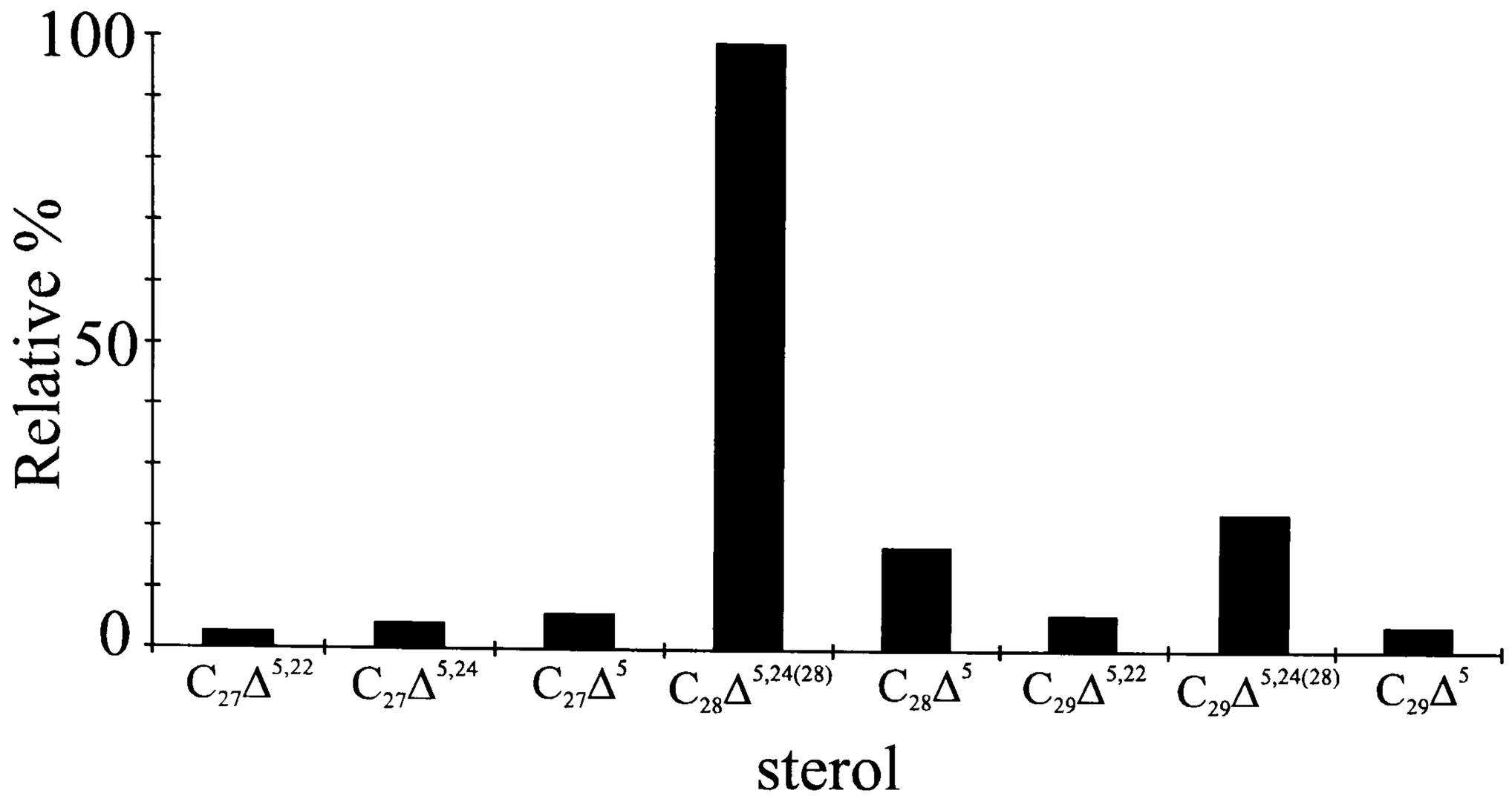


Figure 4-3. *Relative abundance of SCEs in preliminary T. weiss experiment. (For sterol assignments see large scale experiment section 4.3.7.).*

4.2.2. Sterols

Comparison of the culture free sterol distributions with the SCE sterol distribution was complicated by the use of two different cultures of the diatom on the two consecutive feeding days. A total of six sterols was observed over the two cultures but they were found to have significantly different distributions, with one of the six (24-ethylcholesta-5,24(28)-en-3β-ol, **A7**), being absent from the first sample. Analysis of the pre-starved animals revealed the presence of the three common animal sterols (see Chapters 2 and 3) with cholest-5-en-3β-ol (**A1**) being the most abundant component as expected. Therefore, although a total of 8 sterols was observed in the culture and animals, with molecular weights corresponding to the 8 SCE sterols, it was not possible to compare the three pools due to the differences in the two distributions in the two culture samples.

4.2.3. Summary

This small scale experiment confirmed the result of the preliminary experiment (Harradine *et al.*, 1996b), demonstrating the production of SCEs during herbivory and the incorporation of all available algal and animal sterols into SCEs. The practical difficulties encountered during this experiment were addressed and in all future experiments aliquots from only a single culture were used throughout each experiment to provide a better control on culture sterol distributions. The practice of methylation, used to eliminate problems encountered in the resolution of the pigments containing free acid groups (e.g. pyrophaeophorbide *a* [**X**], chls c [**III, IV, V**]), was abandoned after this experiment and an improved analytical protocol was developed for all subsequent experiments using an ODS 3 HPLC column to provide greater resolution than that observed with the ODS 2 column used during this initial experiment.

4.3. RESULTS: LARGE SCALE EXPERIMENT

4.3.1. Algal Culture

The base peak chromatogram (fig. 4-4) of the *T. weiss* pigments is dominated by the C-13^2 epimers of phaeophytin *a* (peaks 8 and 8', **VIII**).

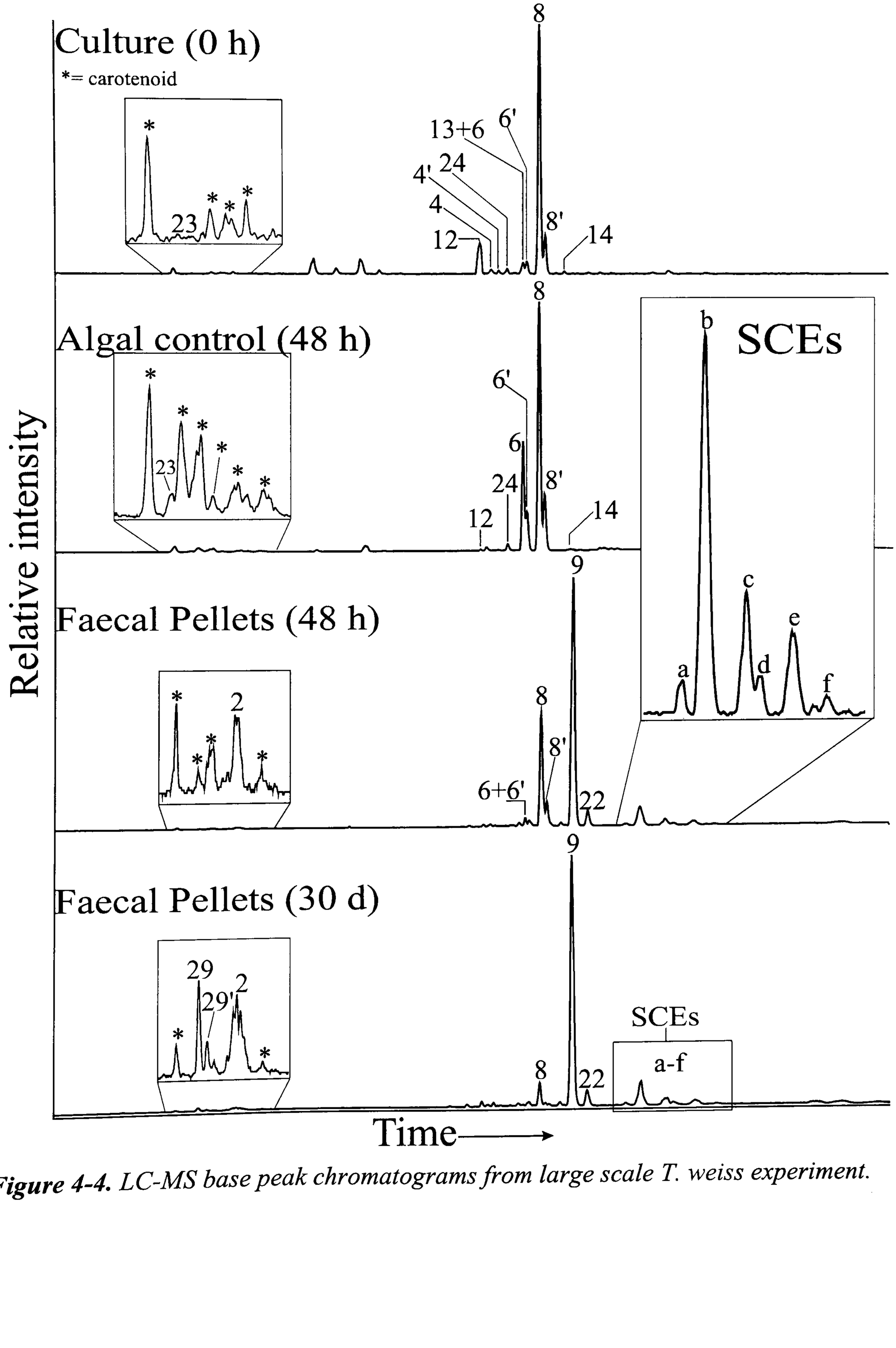


Figure 4-4. *LC-MS base peak chromatograms from large scale T. weiss experiment.*

The C-13^2 mono oxygenated allomer, 13^2-OH phaeophytin *a* (6 and 6', **XIII**), and 15^1-OH phaeophytin *a* lactone (13, **XXXIII**) are present as minor components. Chlorophyll *a* (peaks 4 and 4', **I**) is present in low abundance along with the mono- and di-oxygenated allomers, 13^2-OH chl *a* and 15^1-OH chl *a* lactone (peak 12, **XII** and **XXX**). Also present in trace abundance is phaeophorbide *a* (peak 23, **VII**). This component, only detected during this experiment, reflects the high chlorophyllase content of diatoms (e.g. Jeffrey and Hallegraeff, 1987) compared to the other types of algae using during the wider study in this thesis. A minor chl *a*-like component (peak 24) was observed but could not be identified from its mass spectral data (fig. 4-5) although the ions at *m/z* 642 and 624 might suggest the presence of an OH substituent. Purpurin-18-phytyl ester (14, **XIVa**) was also present (*cf.* Chapters 2 and 3). A number of carotenoids were observed but were not investigated further.

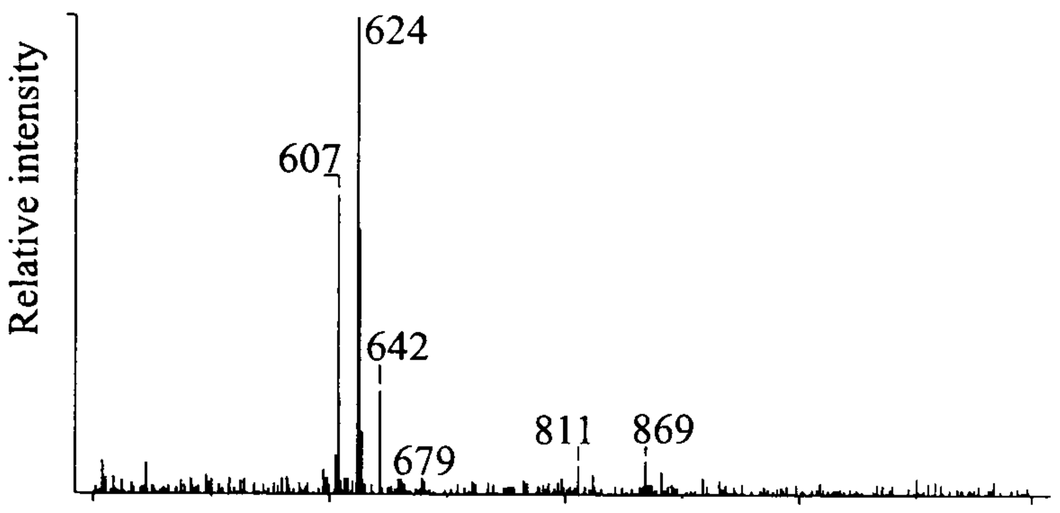


Figure 4-5. *Mass spectrum of peak 24.*

The minor peaks eluting in the SCE region (Fig.4-4) were found to have chlorin-like electronic spectra but mass chromatography did not reveal the presence of typical SCE MH^+ ion peaks or the presence of pyrophaeophorbide *a* (*m/z* 535).

4.3.2. Algal Control

The pigment distribution (fig. 4-4) was again dominated by the C-13^2 epimers of phaeophytin *a* (8 and 8', **VIII**) with a larger proportion of 13^2-hydroxyphaeophytin *a* (6 and 6', **XIII**) relative to the culture. Phaeophorbide *a* (23) and purpurin-18-phytyl ester (14) and the unknown component peak 24 were again present as minor components. The number of carotenoids observed increased suggesting that that a degree of alteration had taken place over the 48h period.

4.3.3. Fresh Faecal Pellets (0 d)

The base peak trace of the bulk (4 beakers, fig. 4-1) pellet sample taken directly after the 48h feeding period (fig. 4-4) shows that the most abundant component is pyrophaeophytin *a* (peak 9, **XI**) with phaeophytin *a* (8, 8') also being major components. Pyrophaeophorbide *a* (2, **X**) is present in trace abundance. Peak 22 is a pyrophaeophytin *a*-like component, tentatively assigned as either pyrophaeophorbide *a* dihydrophytyl ester (**XXXXI**) or mesopyrophaeophytin *a* (**XXXXII**). This component was also observed in the faecal pellets produced when *C. helgolandicus* was allowed to graze on *P. carterae* (Chapter 3).

4.3.4. Faecal Pellets (10 d)

The pellets aged for 10 d (not shown in fig. 4-4, see fig. 4-6) were again dominated by pyrophaeophytin *a* (9) with a lower abundance of phaeophytin *a* (8 and 8') and with pyrophaeophorbide *a* (2) and peak 22 also present. A number of other chl *a* transformation products were also present at all three stages of ageing, but, as electronic data are only available from the 10 d sample due to the hard disk failure, their mass and electronic spectral characteristics are discussed here.

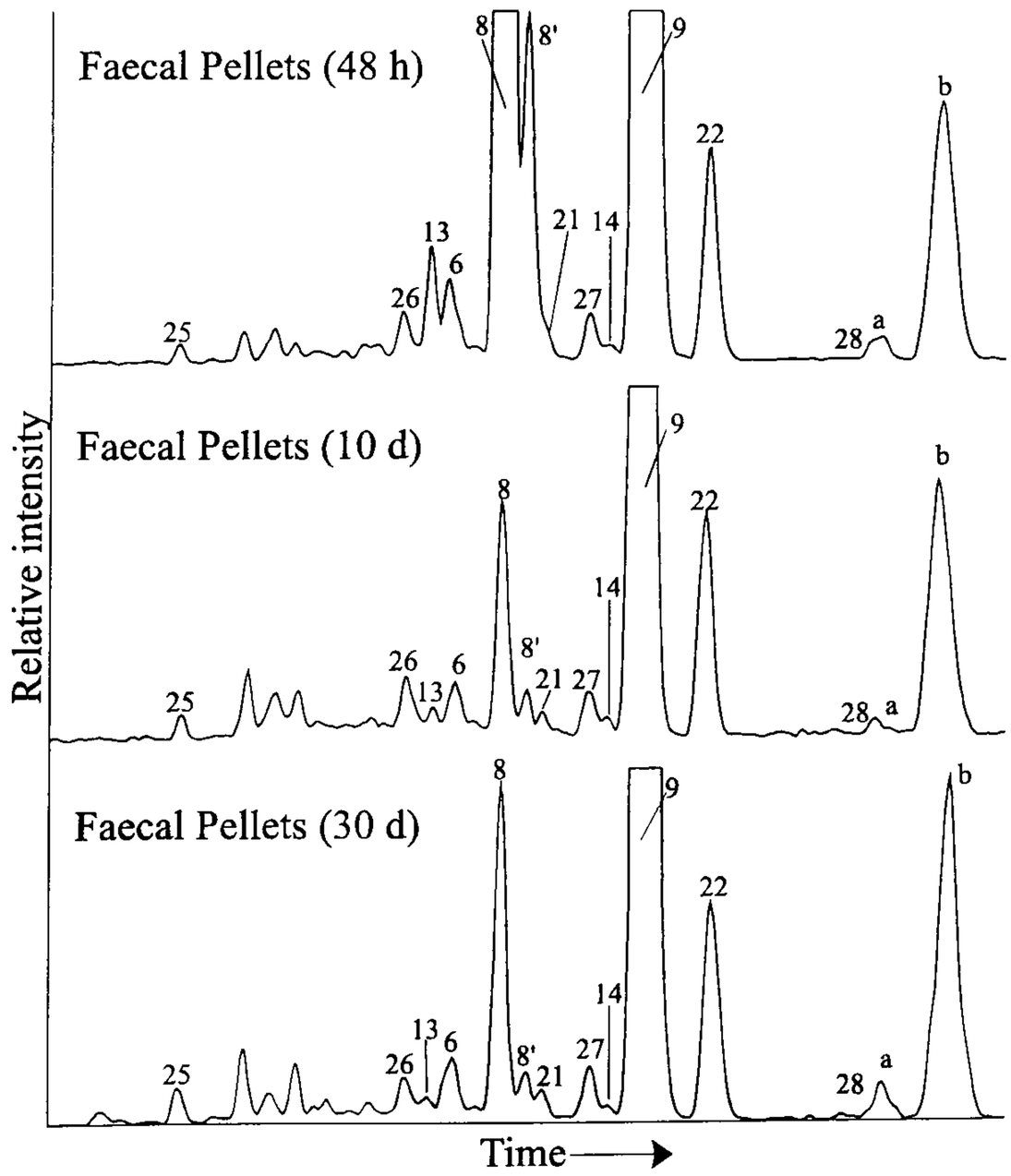


Figure 4-6. *Base peak chromatograms of bulk (4 beaker) pellet samples highlighting minor chlorin components present at all stages of ageing.*

Peak 25 has MH^+ at *m/z* 847 (fig. 4-9) and a fragment ion at *m/z* 569 indicating loss of $C_{20}H_{38}$ (phytadiene). The MH^+ is 34 amu higher than that of pyrophaeophytin *a* which suggests the addition of the elements of H_2O_2. The ions at *m/z* 551 and 535 are therefore assigned as consecutive losses from *m/z* 569 of H_2O and OH with the addition of H. A possible structure for this novel component based on the mass spectral data only is shown in Figure 4-7 (see also discussion). The electronic spectrum ($\lambda_{max.}$ 407, 500, 536, 602 and 659 nm) is similar to that of pyrophaeophytin *a*, however, thereby refuting the assignment as the structure shown (Fig. 4-7) as the ring open component would have a significantly different electronic spectrum to that of pyrophaeophorbide *a* therefore the peak could not be assigned.

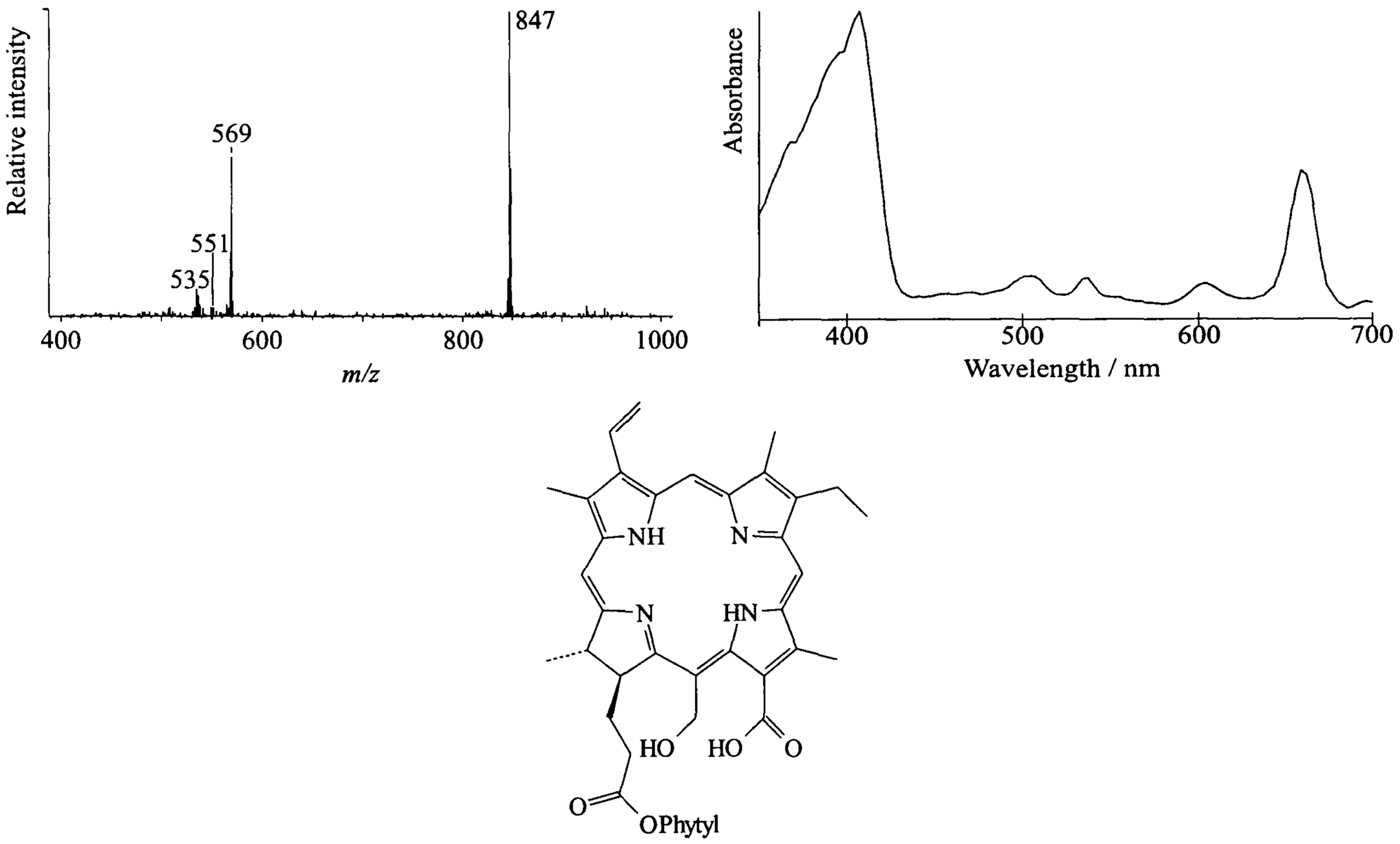


Figure 4-7. *Mass and electronic spectra and proposed structure of peak 25 based on mass spectral data only.*

The electronic spectrum of peak 26 does not show an absorbance maximum in the red region suggesting a porphyrin rather than chlorin type structure. The chls c_1 and c_2 have previously been found to be present in this organism (e.g. Harradine *et al.*, 1996b); however the position of the Soret band (410 nm) does not correspond to that of known chl *c* derivatives (e.g. pyrophaeoporphyrin, Soret $\lambda_{max.}$ 434 nm on-line; Harradine and Maxwell, 1998). The mass spectrum showed only one significant ion at *m/z* 829 with a

minor ion at *m/z* 945 neither of which could be recognised in terms of a possible structure, so the component could only be assigned as chl *c*-like.

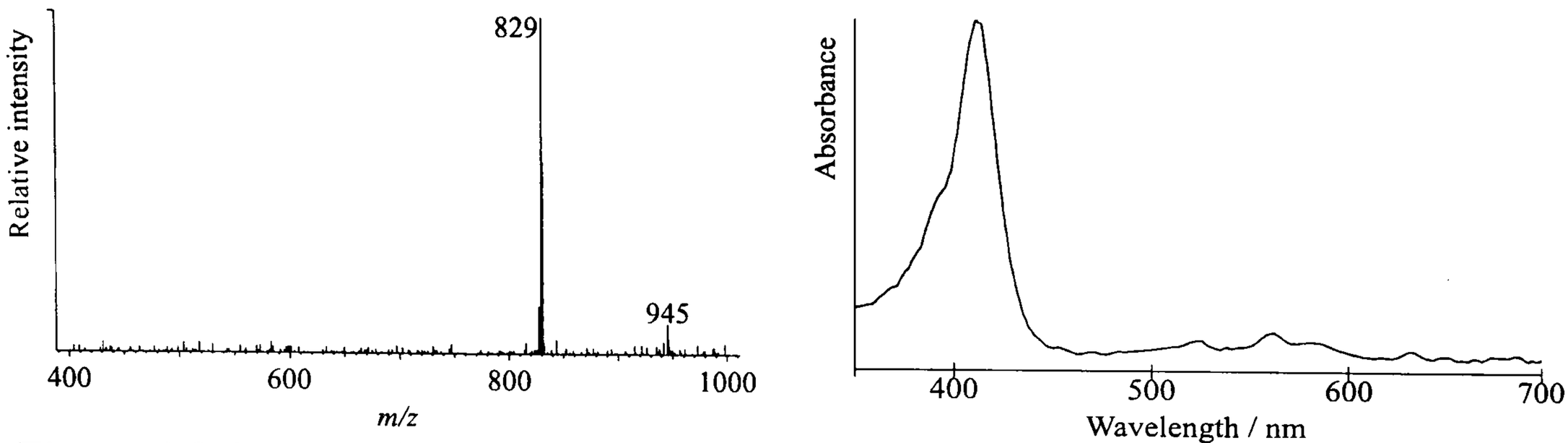


Figure 4-8. *Mass and Electronic spectra of peak 26.*

Peak 27, eluting just prior to purpurin-18-phytyl ester (14), is tentatively assigned as containing the 13^2-oxopyrophaeophorbide *a* (**XXXXIIIa**) nucleus (Watanabe *et al.*, 1993; Sakata *et al.*, 1994). It has an unusual electronic spectrum ($\lambda_{max.}$ 386, 419, 512, 629, 674 nm; fig. 4-9) comparable with that reported for 13^2-oxopyrophaeophorbide *a* ($\lambda_{max.}$ 288, 386, 514, 618, 676 nm in MeOH; Watanabe *et al.*, 1993) however there was no mention of the split Soret band by the authors; this was noted, however, for the enolate form (Kozyrev, 1998). Watanabe *et al.* (1993) also performed mass spectral analysis and found MH^+ at *m/z* 549 which is present in the mass spectrum of peak 27 (fig. 4-9). Therefore, taking MH^+ as *m/z* 827 (fig. 4-9), loss of $C_{20}H_{38}$ to give *m/z* 549 suggests that this component is the phytyl ester of 13^2-oxopyrophaeophorbide *a* (fig. 4-9). The ions at *m/z* 521 and 503 are assigned as consecutive losses of CO and H_2O respectively.

A minor component (peak 28) was also observed in all pellet samples, partially co-eluting with the earliest eluting SCE (fig. 4-6 and see below). It has a similar electronic spectrum ($\lambda_{max.}$ 386, 422, 518, 617, 674 nm; fig. 4-10) to peak 27, again showing the split Soret band, and a similar mass spectrum containing a fragment ion at *m/z* 549. In this case the MH^+ at *m/z* 929 suggests loss of $C_{28}H_{44}$. This indicates that the alcohol is a C_{28} sterol containing 2 double bonds and is assigned as the most abundant algal sterol (see below), 24-methylcholest-5,24(28)-dien-3β-ol (**A4**).

Peak 21, also found in the *P. carterae* experiment in Chapter 3, is tentatively assigned as pyrophaeophorbide *a*-dehydrophytyl ester (**XXXXI**). Purpurin-18-phytyl ester (peak 14, **XIVa**) was also present in all samples.

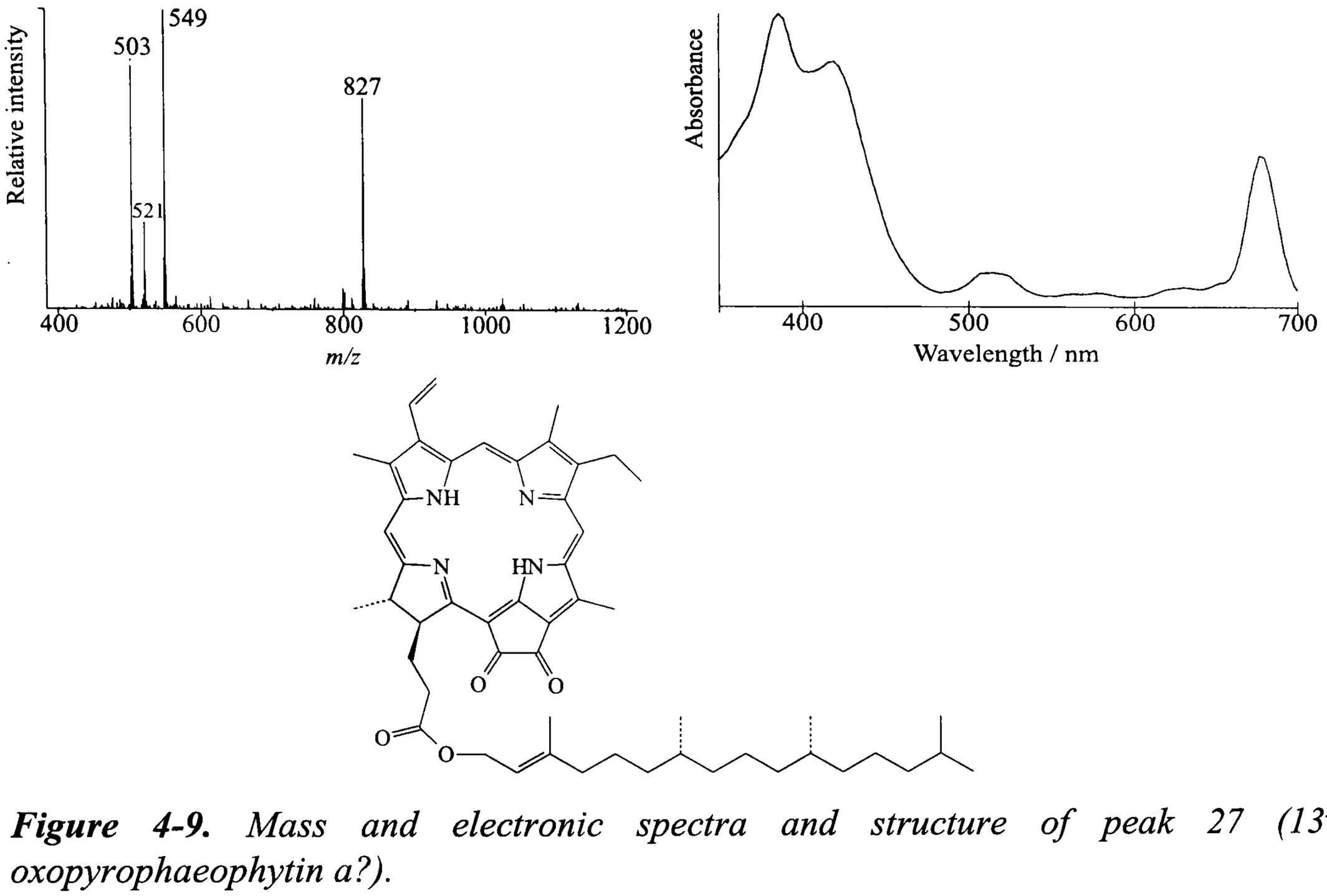


Figure 4-9. *Mass and electronic spectra and structure of peak 27 (13^2-oxopyrophaeophytin a?).*

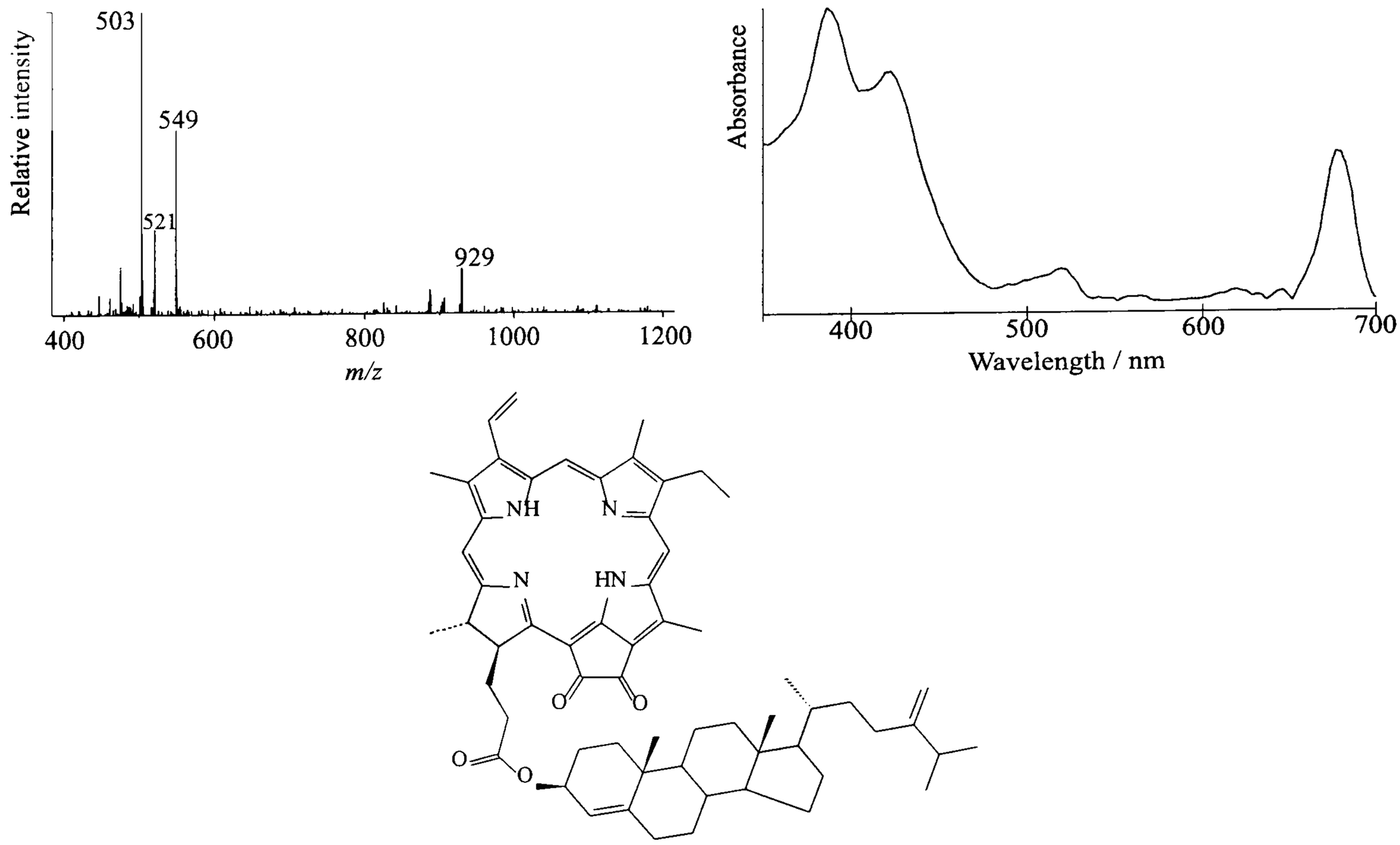


Figure 4-10. *Mass and electronic spectra and structure of peak 28 (13^2-oxopyrophaeophorbide a-24-methylcholesta-5,24(28)-dien-3β-ol?).*

4.3.5. Faecal Pellets (30 d)

The abundance of phaeophytin *a* (8 and 8'; fig. 4-4) is greatly reduced relative to that of pyrophaeophytin *a* (9) in the pellets aged for 30 d. As well as the components described above, mass chromatography (fig. 4-11) revealed the presence of the C-13^2 *R* and *S* epimers of 13^2-hydroxychlorophyllone *a* (chlorophyllone [**XVI**]; e.g. Sakata *et al.*, 1990) in the samples aged for 10 d and 30 d. The mass spectra (available from 30 d sample only; fig. 4-12) of these components (peaks 29 and 29') show MH^+ at *m/z* 533 with fragment ions at *m/z* 515 (loss of H_2O) and *m/z* 489. Chillier *et al.* (1993) observed a fragment at *m/z* 490 by desorption mass spectrometry in EI mode, which they assigned as loss of ketene (CH_2CO) from the molecular ion at *m/z* 532 due to a temperature effect; they did not see this ion, however, in the FAB-spectrum. It is therefore suggested that in this case the ion at *m/z* 489 is due to the loss of C_2H_4O. The ion at *m/z* 581, abundant in the spectrum of the S isomer (fig. 4-12b) is thought to be due to co-elution of an unknown component with this isomer.

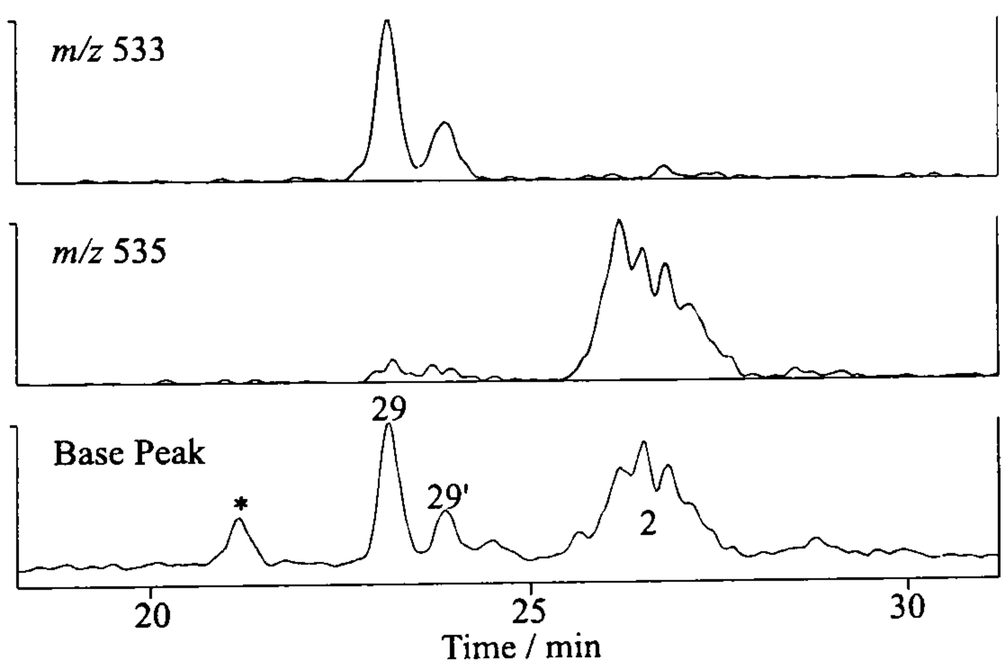


Figure 4-11. *Mass chromatograms showing relative retention times of the R and S C-13^2 epimers of 13^2-hydroxychlorophyllone a (peaks 29 and 29') and pyrophaeophorbide a (peak 2).*

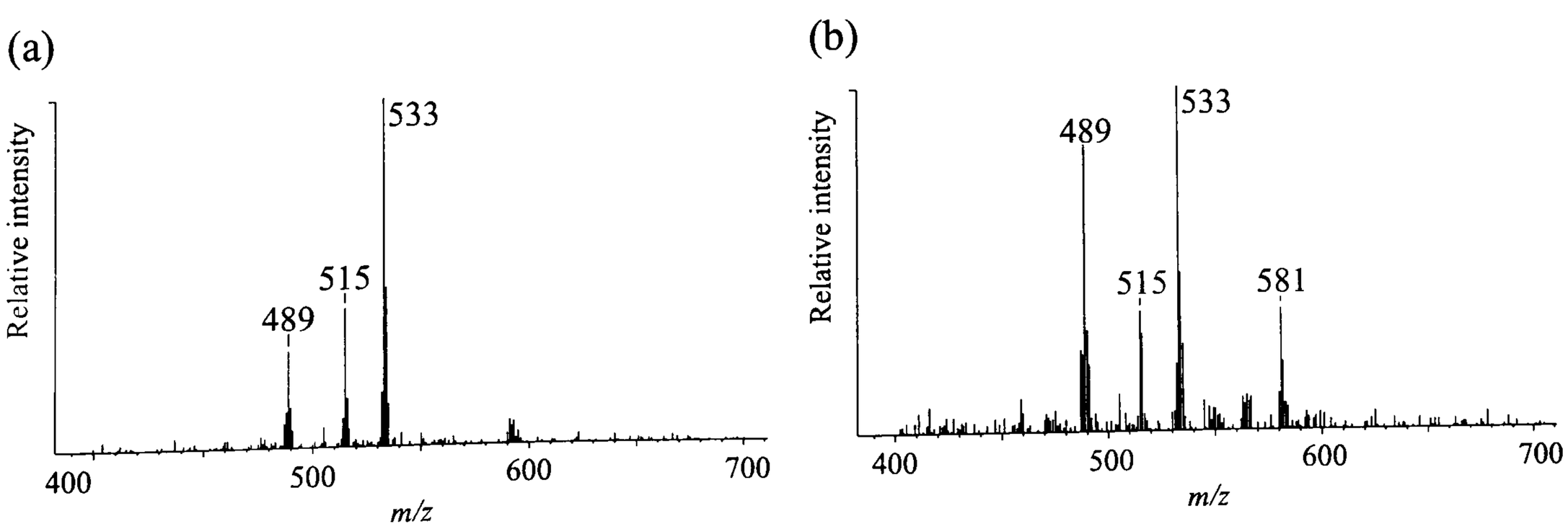


Figure 4-12. *Mass spectra of (a) peak 29 (13^2-OH chlorophyllone a) and (b) peak 29' (13^2-epi-OH chlorophyllone a).*

4.3.6. SCEs

A total of six peaks (a-f) were observed in the SCE region in all pellet samples (fig. 4-4). Mass chromatography (fig. 4-13) revealed a total of 8 SCEs containing C_{27}-C_{29} mono and diunsaturated sterol moieties (*cf.* Table 4-1). It should be noted that the same number of SCEs with identical MH^+ values were identified in the first stage small scale experiment (fig. 4-2).

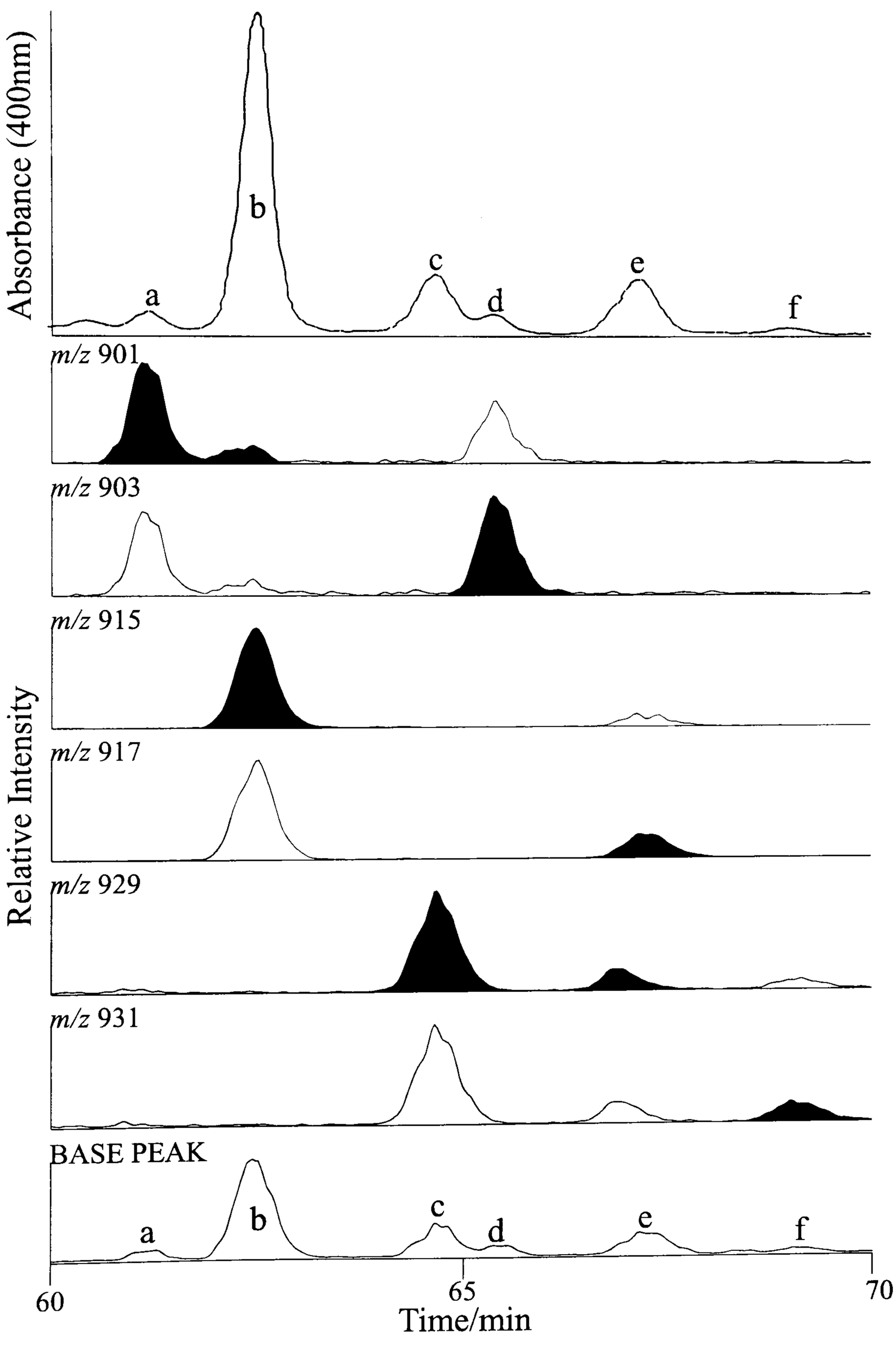


Figure 4-13. *Absorbance (400 nm) and mass chromatograms of faecal pellet SCE region (10 d sample).*

The SCEs (peaks a-f) all have electronic spectra similar to that of pyrophaeophorbide *a* and mass spectra of the individual peaks (figs. 4-14 to 4-19) show the expected fragment ion at *m/z* 535. The minor ion at *m/z* 929 in Figure 4-14 is due to partial co-elution with peak 28 (see fig. 4-6) and the same ion in Figure 4-17 from the same effect with peak c. The unusual doublet between 650 and 700 nm in Figure 4-17 also suggests co-elution of another component which could not be identified from the available data.

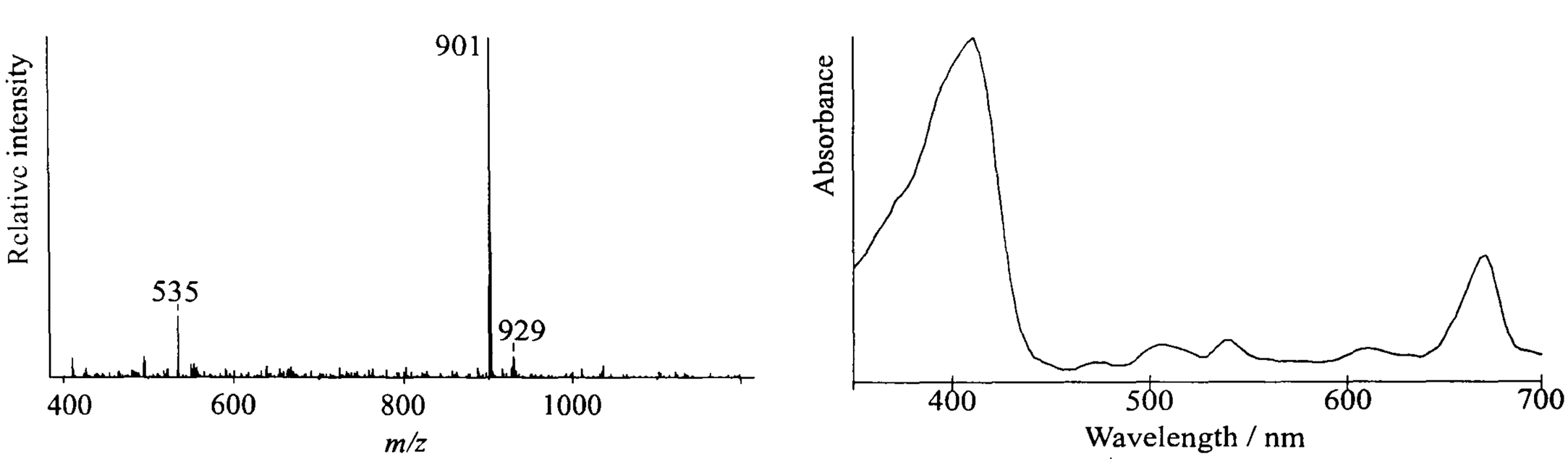


Figure 4-14. *Mass and electronic spectra of SCE peak* ***a*** *(MH^+ = 901).*

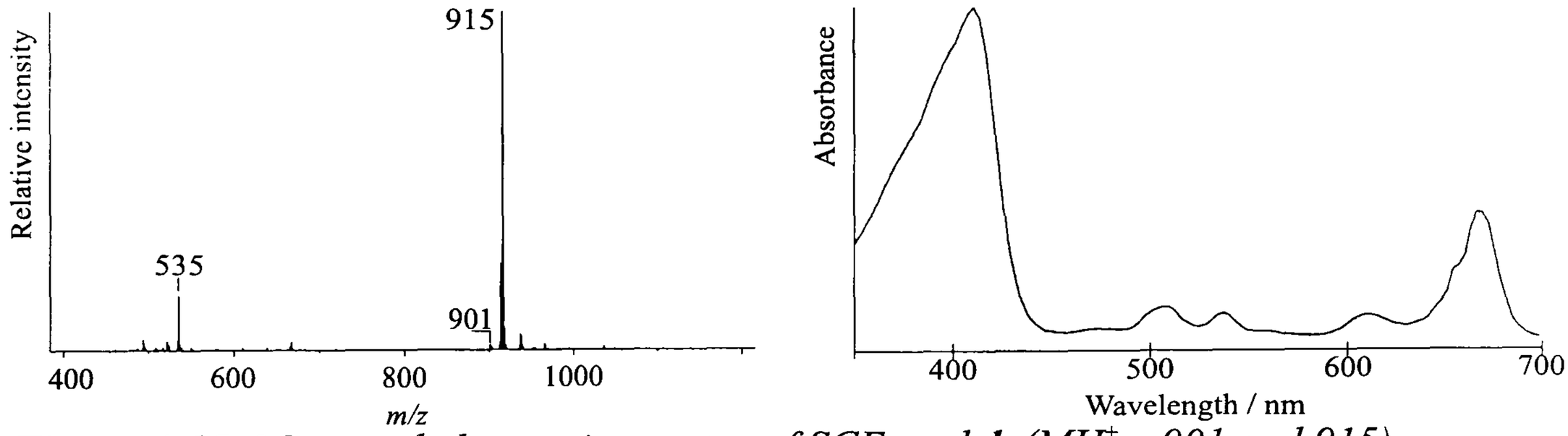


Figure 4-15. *Mass and electronic spectra of SCE peak* ***b*** *(MH^+ = 901 and 915).*

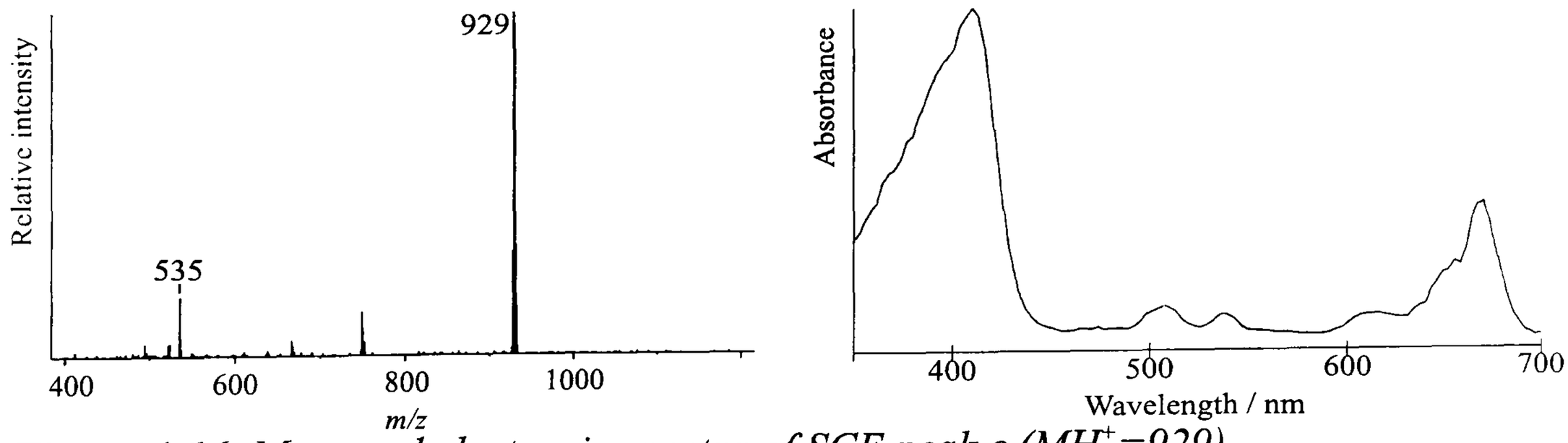


Figure 4-16. *Mass and electronic spectra of SCE peak* ***c*** *(MH^+=929).*

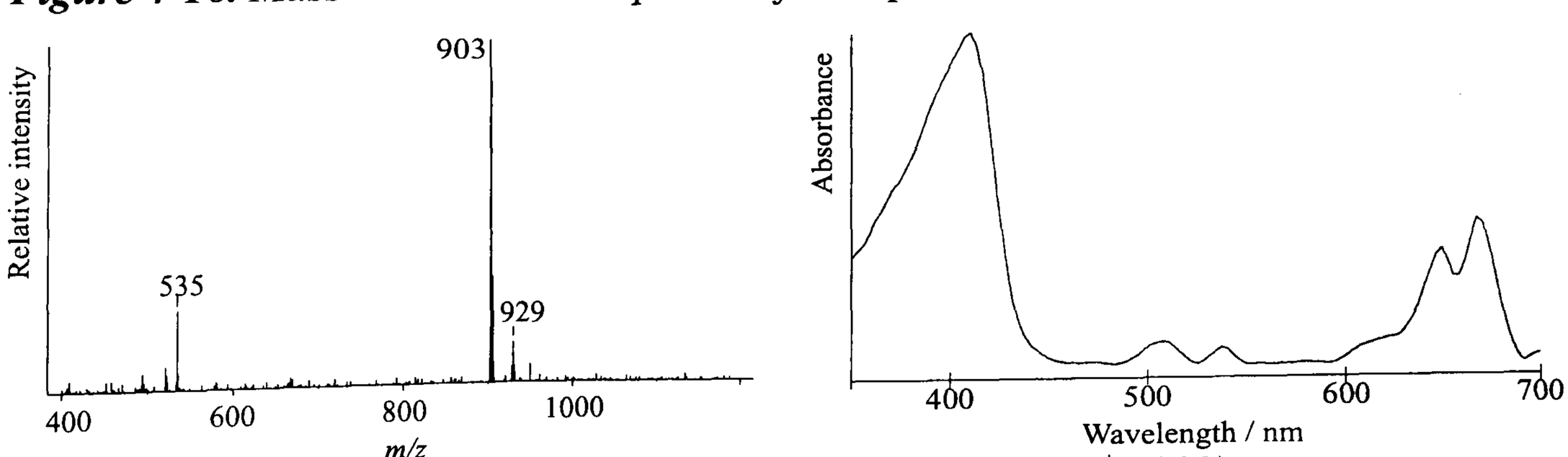


Figure 4-17. *Mass and electronic spectra of SCE peak* ***d*** *(MH^+=903).*

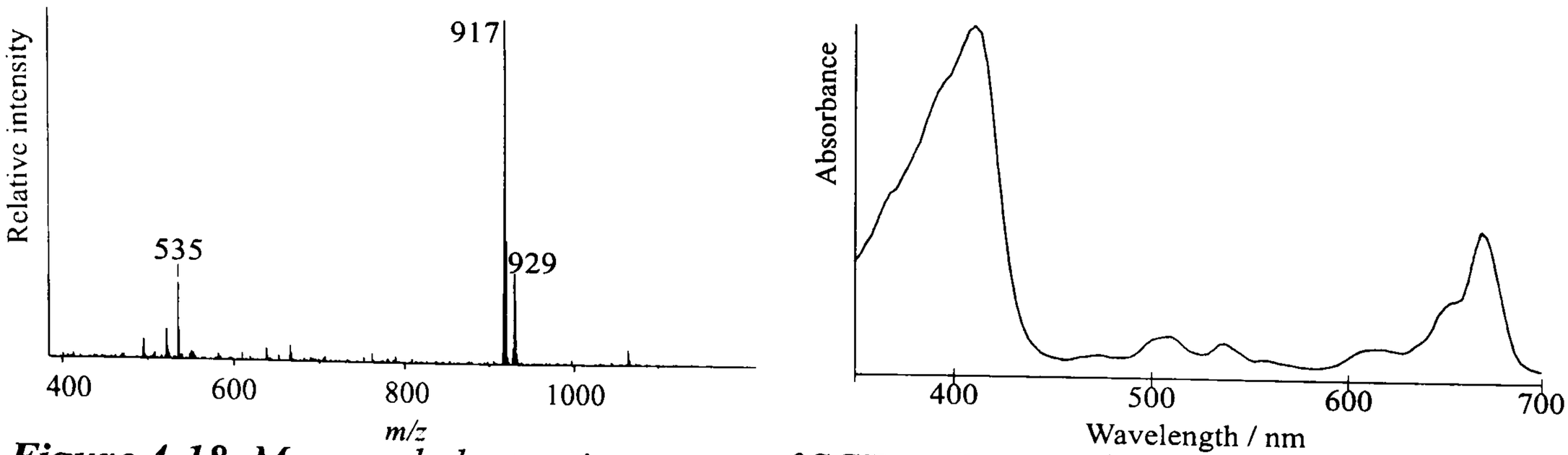


Figure 4-18. *Mass and electronic spectra of SCE peak* ***e*** *(MH^+=917 and 929).*

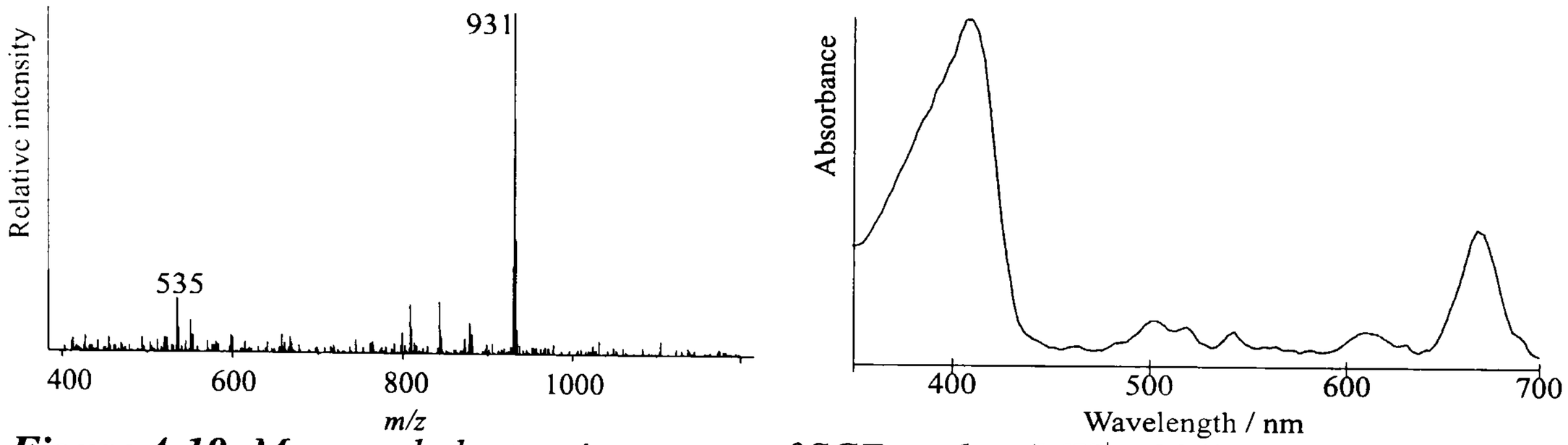


Figure 4-19. *Mass and electronic spectra of SCE peak* ***e*** *(MH^+=931).*

4.3.7. Free Sterols

4.3.7.1. Starved Animals

The starved animal sterol distribution (fig. 4-20a) contained the three usual animal sterols cholest-5-en-3β-ol (**A1**), cholesta-5,24-dien-3β-ol (**A2**) and cholesta-5,22-dien-3β-ol (**A3**); however, also present were four phytosterols 24-ethylcholest-5-en-3β-ol (**A8**), 24-ethylcholesta-5,24(28)-dien-3β-ol (**A7**), 24-ethylcholesta-5,22-dien-3β-ol (**A9**) and 24-methylcholesta-5,24(28)-dien-3β-ol (**A4**) indicating that in this case insufficient time had passed to allow the animals to fully clear their guts. As expected cholest-5-en-3β-ol was the most abundant sterol, comprising *ca.* 50% of the total.

4.3.7.2. Culture

The algal distribution (fig. 4-20b) is dominated by 24-methylcholesta-5,24(28)-dien-3β-ol (**A4**), comprising *ca.* 70% of the total. This sterol is commonly observed to be amongst the most abundant in centric diatoms along with 24-methylcholest-5-en-3β-ol (**A5**) and cholest-5-en-3β-ol (**A1**) (Barrett *et al.*, 1995). Pennate diatoms generally have a more diverse sterol distribution although it is usually dominated by a single sterol. Other minor components were 24-methylcholest-5-en-3β-ol (**A5**), 24-ethylcholest-5-en-

3β-ol (**A8**), 24-ethylcholesta-5,24(28)-dien-3β-ol (**A7**), 24-ethylcholesta-5,22-dien-3β-ol (**A9**) and cholest-5-en-3β-ol (**A1**).

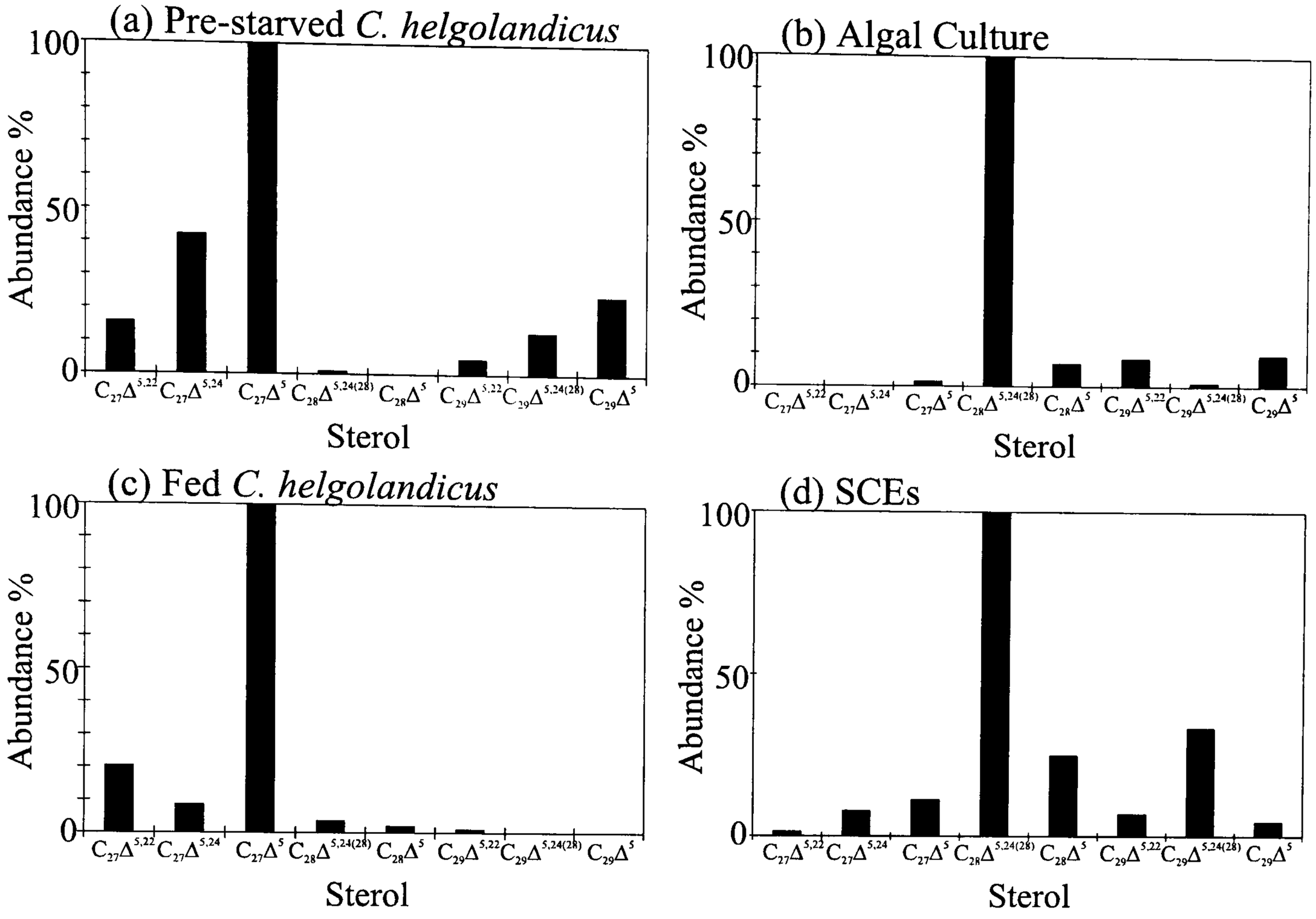


Figure 4-20. *Free and SCE sterol relative abundances.*

4.3.7.3. Fed Animals

The fed animal sterol distribution (fig. 4-20c) was slightly simpler than that of the starved animals again with the three expected sterols being the most abundant components and with minor contributions of 24-methylcholesta-5,24(28)-dien-3β-ol (**A4**), 24-methylcholest-5-en-3β-ol (**A5**) and 24-ethylcholesta-5,22-dien-3β-ol (**A9**). The relative abundances of cholest-5-en-3β-ol and cholesta-5,22-dien-3β-ol are similar to that in the starved animals but the abundance of cholesta-5,24-dien-3β-ol is significantly reduced (*cf.* Chapters 2 and 3; see also discussion).

4.3.7.4. Comparison of SCE and Free Sterol Distributions

Analysis of the starved animals and the algal culture revealed the presence of 8 C_{27}-C_{29} mono and diunsaturated sterols, directly matching those predicted by the SCE analysis (*cf.* Table 4-1). The animal C_{27} sterols were assigned as described previously (Chapter

2). SCE peak e was assigned as the 24-ethylcholesta-5,22-en-3β-ol (**A9**) ester by co-injection of a synthesised standard (Pearce *et al.*, 1993) so peak c by default was therefore assigned as the 24-ethylcholesta-5,24(28)-dien-3β-ol (**A7**) ester. The other SCE MH^+ values corresponded to a single available sterol and were assigned accordingly (table 4-2).

SCE Peak*	Esterifying Sterol	Sterol Structure
a	cholesta-5,24-dien-3β-ol	**A2**
b	cholesta-5,24-dien-3β-ol	**A3**
	24-methylcholesta-5,24(28)-dien-3β-ol	**A4**
c	24-ethylcholesta-5,22-dien-3β-ol	**A9**
d	cholest-5-en-3β-ol	**A1**
e	24-methylcholest-5-en-3β-ol	**A5**
	24-ethylcholesta-5,24(28)-dien-3β-ol	**A7**
f	24-ethylcholest-5-en-3β-ol	**A8**

Table 4-2. *SCE sterols and structures (* see figs. 4-4 and 4-13).*

Comparison of the starved animal, algal and SCE sterol distributions (figs. 4-20a,b,d) shows that some alteration of the available sterol distributions has occurred prior to esterification although the situation is complicated due to the presence of some of the same phytosterols found in the alga also being present in the pre-starved animals. The three common animal sterols (fig. 4-20a; **A1,2,3**) were present in the SCE fraction in broadly comparable relative abundances to that in the pre-starved animals (fig. 4-20d). As the pre-starved animals still contained residual amounts of phytosterols and sterol assimilation is occurring (see below) it is difficult to estimate accurately the relative contributions of diatom and animal sterols to the SCEs. It is clear, however, that the SCE sterols arise mainly from the diatom (fig. 4-20b,d), so if the contributions of 24-ethylcholesta-5,22-dien-3β-ol (**A9**), 24-ethylcholesta-5,24(28)-dien-3β-ol (**A7**) and 24-ethylcholest-5-en-3β-ol (**A8**) from the pre-starved animals (fig. 4-20a) to the SCEs is ignored, then *ca*. 90% of the SCE sterols derive from the diatom sterols.

4.3.8. Ageing Studies

Comparison of the relative abundances of the SCE sterols at the three stages shows that there is no change in the distribution over the 30 day period (fig. 4-21).

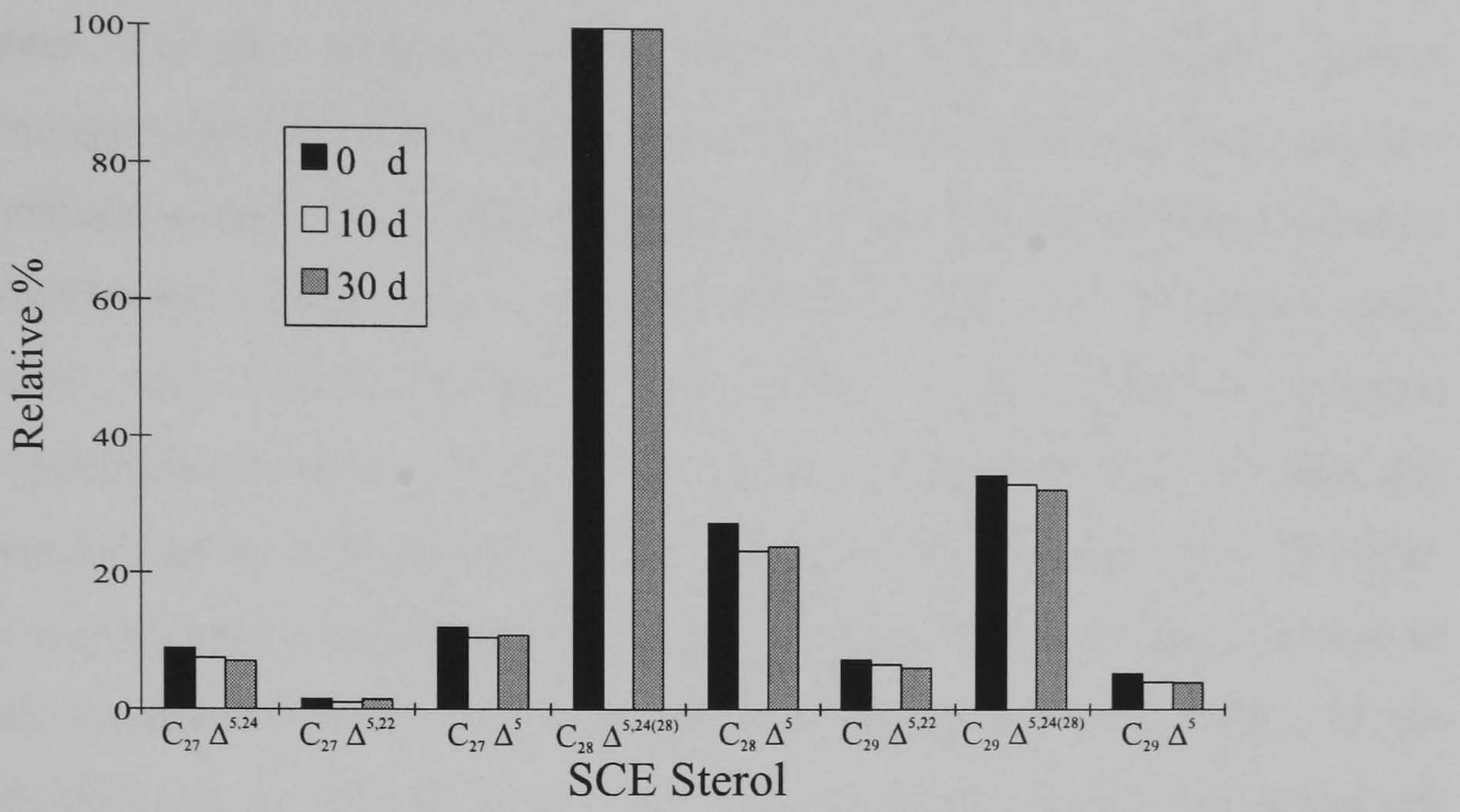


Figure 4-21. *Faecal pellet SCE relative abundance with ageing (based on LC-MS mass chromatogram MH^+ peak areas).*

Although the relative distribution of the SCEs remained constant during ageing the total mass of SCEs per pellet fell from *ca.* 0.9 μg (day 0) to 0.7 μg (day 10) and 0.3 μg (day 30), an overall loss of *ca.* 70% (fig, 4-22a); however, the mass of SCEs relative to total chlorins was observed to approximately double, increasing from *ca.* 8.5 % (day 0) to 16% (day 30) (fig. 4-22b).

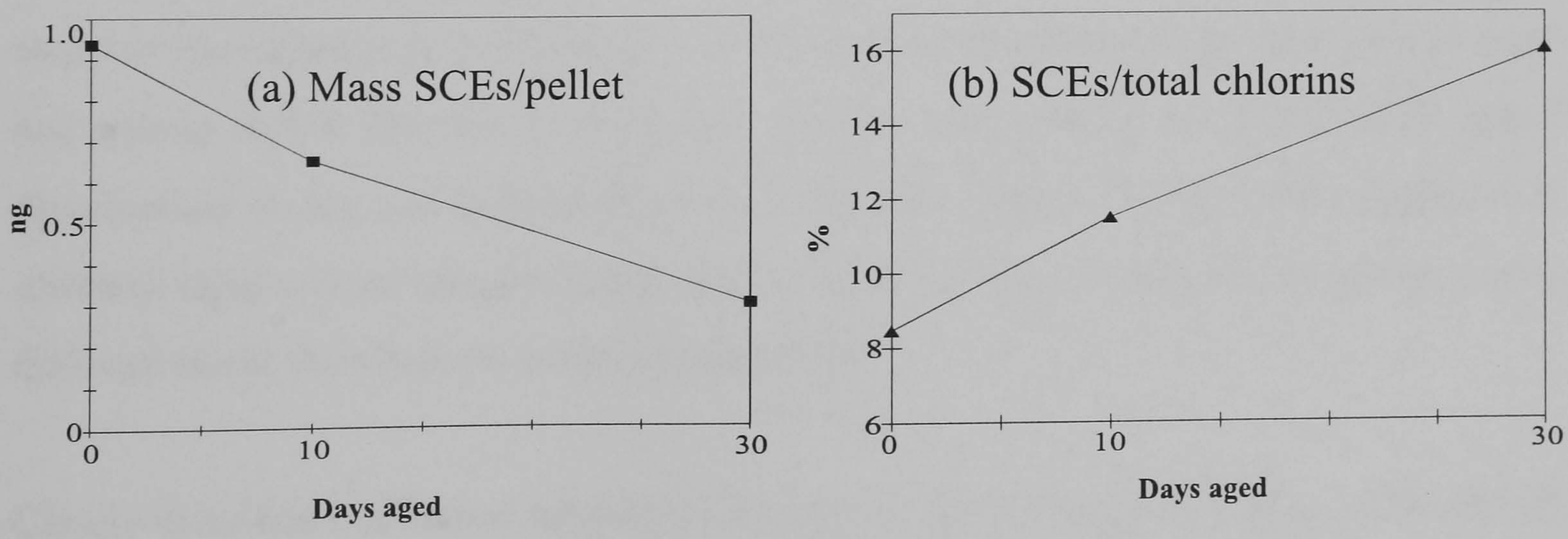


Figure 4-22. *(a) mass of SCE per pellet, (b) SCEs % of total chlorins.*

4.4. DISCUSSION

The production of SCEs has been demonstrated during two experiments in which the copepod *C. helgolandicus* was allowed to graze on *T. weissflogii*, a commonly occurring marine diatom. Previous studies (Downs, 1989) involving the copepod *Calanus pacificus* grazing on the same diatom demonstrated the production of the same common herbivory products as observed in this study (phaeophytin *a* [**XIII**], pyrophaeophytin *a* [**XI**], phaeophorbide *a* [**XII**] and pyrophaeophorbide *a* [**X**]). In the earlier study, however, there was significantly higher production of phaeophorbide products particularly pyrophaeophorbide *a* (assigned by Downs as phaeophorbide *a*3) than that observed here. In a series of experiments at different algal cell concentrations (750-6000 cells ml^{-1}) it was observed that at higher food concentrations the extent of conversion of phaeophytins to phaeophorbides was significantly reduced (Downs, 1989). In the present study the high cell concentration used (13000 cells ml^{-1}) could explain the low production of pyrophaeophorbide *a* and absence of phaeophorbide *a* from the faecal pellets. As it is not known if the mechanism of SCE formation occurs *via* transesterification of pyrophaeophytin *a* or esterification of pyrophaeophorbide *a* it is not possible to predict whether SCE production would be greater at low food concentrations, where there is greater production of pyrophaeophorbide *a*, or at high food concentrations where there is greater production of pyrophaeophytin *a*.

These two experiments performed here with the same organisms used in the preliminary study of Harradine *et al.* (1996b) have confirmed the incorporation of all available algal and animal sterols into the SCE fraction. Perhaps surprisingly, the overall SCE sterol distributions in the two experiments are comparable (figs. 4-3 and 4-20d) despite two different algal culture samples being used in the first stage experiment, resulting in two different sterol distributions in the copepod food.

Clearly there has been some alteration of the algal sterol distribution (fig. 4-20b and d) prior to esterification (*cf.* Chapter 3). Particularly evident is the decrease in abundance of 24-methylcholesta-5,24(28)-dien-3β-ol (**A4**) relative to 24-methylcholest-5-en-3β-ol (**A5**) and 24-ethylcholesta-5,24(28)-dien-3β-ol (**A7**). This difference is attributed to the

utilisation of the C-24 alkylated sterols for the production of cholest-5-en-3β-ol (**A1**) *via* cholesta-5,24-dien-3β-ol (**A2**) (Goad, 1978, 1981; see also Chapter 2).

This is the first experiment (*cf.* Chapters 2, 3) in which cholest-5-en-3β-ol was present in the algal substrate; however, its abundance was so low (<2%) relative to that of 24-methylcholest-5-en-3β-ol as to be negligible so the cholesterol SCE component is thought to derive mainly from the animal itself or from assimilation of other sterols. The apparent reduction in the abundance of cholesta-5,24-dien-3β-ol in the fed animals relative to that of the starved animals (fig. 4-20a, b) suggests that the animal was utilising its store of this sterol, perhaps during a period of acclimatisation to the new feeding regime following the period of starvation (*cf.* Chapter 2). This indicates, therefore, that the extent of modification of algal sterol distributions prior to incorporation into SCEs is dependent on both the presence and abundance of cholest-5-en-3β-ol in the diet and possibly also on the previous feeding history of the animals which determines their cholest-5-en-3β-ol and cholesta-5,24-dien-3β-ol content at the commencement of feeding. Bradshaw *et al* (1989) noted that certain marine invertebrates including crustaceans appear to assimilate C_{28} sterols more readily than C_{29} sterols. This is clearly observed here for the C_{28} and C_{29} $\Delta^{5,24(28)}$ components (fig. 4-20b, d) which are structurally comparable as intermediates in the pathway linking the dealkylation of C-24 substituted components to the production of cholesterol (fig. 4-23). However, this does not seem to be the case for C_{28} and C_{29} Δ^{5} components and rather it appears that the C_{29} Δ^{5} component has been converted to C_{29} $\Delta^{5,24(28)}$, increasing the relative abundance of the latter prior to esterification.

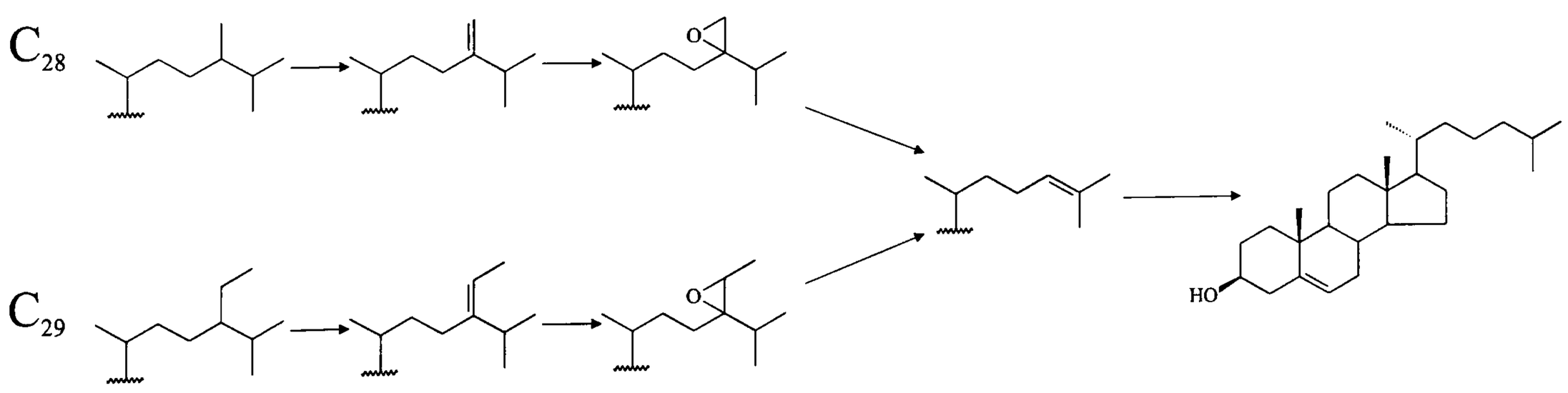


Figure 4-23. *Dealkylation pathway for conversion of C-24 substituted sterols to cholest-5-en-3β-ol.*

The SCE sterol distribution (fig. 4-21) was observed to be unaltered during pellet ageing (30 d). This suggests that, although there is clearly alteration of substrate sterol distributions prior to esterification, once esterified the SCE sterol distribution is consistent, at least for a period of one month. Therefore, assuming an average pellet sinking rate of *ca.* 100 m d^{-1} (Corner *et al.*, 1986) pellets produced in surface waters could perhaps reach a depth of up to *ca.* 3000 m with the SCE sterol distribution still intact. Pellet sinking rate is dependent on a number of factors, but dimensions and density would seem to be the most important factors; hence, larger pellets produced by larger zooplankton such as adult copepods and macrozooplankton and particularly pellets containing diatom frustules (Voss, 1991) or haptophyte coccoliths (e.g. Harris, 1994) have been found to have higher sinking rates than those containing naked flagellates, thereby ensuring removal from the photic zone before pigments can be destroyed by photooxidation (*cf.* Caron *et al.*, 1989). Therefore, the stability of the sterol distribution coupled with the greater stability of SCEs relative to other chlorins such as phaeophytin *a* adds support to the idea that sedimentary SCE sterols are better indicators of phytoplankton palaeocommunity structure than the corresponding sedimentary free sterols which are susceptible to extensive biodegradation and distributional changes in the water column and bottom sediments (e.g. Gagosian and Heinzer, 1979; Gagosian *et al.*, 1980, 1981; Wakeham, 1989, Teece, 1994; see also Chapters 5 and 7).

The chl *a* transformation product purpurin-18-phytyl ester (**XIVa**) was observed in the large scale experiment at all stages from the algal culture through to the pellets aged for 30 d. In the chlorophyta and haptophyta experiments (Chapters 2 and 3) it was only detected in the faecal pellets; however, as it has been observed to be produced at the earliest stages of alteration in the water column (Naylor and Keely, 1998) it is considered most likely to have been present in amounts below detection limits in the cultures in experiments in Chapters 2 and 3. This component is a product of an oxidative Type I reaction (Chapter 1, section 1.2.4.) in which the chromophore remains intact but modification of the substituents, in this case the exocyclic ring E, occurs with the incorporation of additional oxygen atoms. This type of chlorophyll transformation is important as it is considered to be the an early stage in the conversion of chls to aetioporphyrins (e.g. aetioporphyrin III, fig 4-24), one of the main sedimentary

porphyrin types, in which the exocyclic ring is absent (e.g. Naylor and Keely, 1998 and references therein).

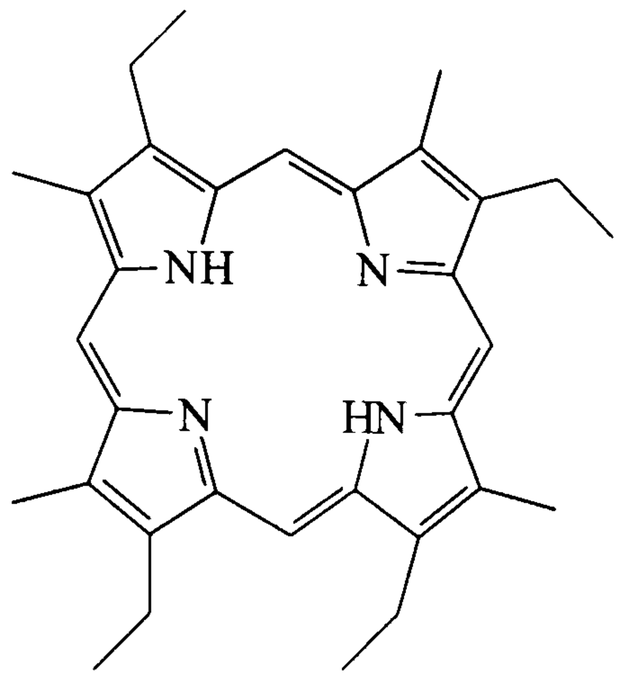


Figure 4-24. *Aetioporphyrin III.*

Initially, aetioporphyrins were thought to be formed by thermolytic cleavage of the exocyclic ring E in cycloalkanoporphyrins (CAPs), another of the main sedimentary porphyrin types (e.g. DPEP, **VI**); however it is now thought more likely that they originate, at least in part, from the oxidative cleavage of the exocyclic ring of chlorophylls. The reaction is initiated by incorporation of oxygen at the C-13^2 position, leading to the formation of the mono oxygenated allomer then further oxidation reactions with a variety of intermediates resulting in ring opening and the production of a range of structures.

Recently, two such intermediates, purpurin-18-phytyl ester (**XIVa**) and purpurin-18 (**XIVb**), have been isolated from bottom sediments under water columns oxygenated through to the sediment water interface although they were not present in sediment from a predominantly anoxic lake. Purpurin-18 has also been observed in trace abundance in a clam (Watanabe *et al.*, 1993) where it was thought to act as an antioxidant (by a radical scavenging mechanism), along with a number of other unusual chl transformation products (see below). The occurrence of purpurin-18-phytyl ester (peak 14, fig. 4-4 and 4-6 and see Chapters 2, 3 and 5) in the pellets is thought to be the first report of this compound in zooplankton faecal material. The laboratory experiment therefore provides information on a method of transport of these types of components to bottom sediments and their presence in such sediments. Previously it has been suggested that they arise from the postdepositional diagenetic transformation of chl *a* or its

degradation products (Ocampo and Repeta, 1999 and references therein). This seems surprising in the light of the Naylor and Keely (1998) and present findings (see above).

The two epimers of 13^2-hydroxychlorophyllone *a* (chlorophyllone, **X**), one of the most widely occurring sedimentary chlorins (e.g. Chillier *et al.*, 1993; Harris *et al.*, 1995b), were found in low abundance only in the pellets after ageing. This might suggest, *a priori*, that the formation of chlorophyllone in the natural environment is, like that of the SCEs, a result of herbivory. However, chlorophyllone (**XVI**) was first reported in a number of marine invertebrates and proposed as an antioxidant (Sakata *et al.*, 1990; Watanabe *et al.*, 1993) and was also present in minor amounts in both mixtures and mono cultures of marine diatoms (Sakata *et al.*, 1994) as well as in *Isochrysis galbana* (haptophyte) which was incorrectly assigned as a diatom (Sakata *et al.*, 1994). The invertebrates involved were known to feed on diatoms, so chlorophyllone would seem to originate from chl present in the ingested diatoms metabolised by either the diatoms themselves, organisms symbiotic with the diatoms, enzymes present in the clam or organisms symbiotic with the clam (Watanabe *et al.*, 1993). It is difficult therefore to explain its appearance only in the aged pellets in the present study. It seems likely that it was not observed in the culture or control because it was in low abundance relative to other chlorins but was detected in the pellets as a result of a greater stability relative to other chlorins (*cf.* SCEs). If so, enhanced stability would explain its typical occurrence as a significant chlorin in bottom sediments (e.g. Chillier *et al.*, 1993; Harris *et al.*, 1995b; Harris and Maxwell, unpublished results).

Chlorophyllone is thought to be produced from chl *a via* a pathway (fig. 4-25) involving phaeophorbide *a* and 13^2,17^3-cyclophaeophorbide *a* enol (**XXXXIV**) which has been found to be present in a sponge and to be associated with diatoms symbiotic with the sponge (Karuso *et al.*, 1986); it has also recently been detected as an abundant component of both sediment trap material (Ocampo *et al.*, 1999) and recent sediments (Ocampo *et al.*, 1999; Talbot, unpublished results).

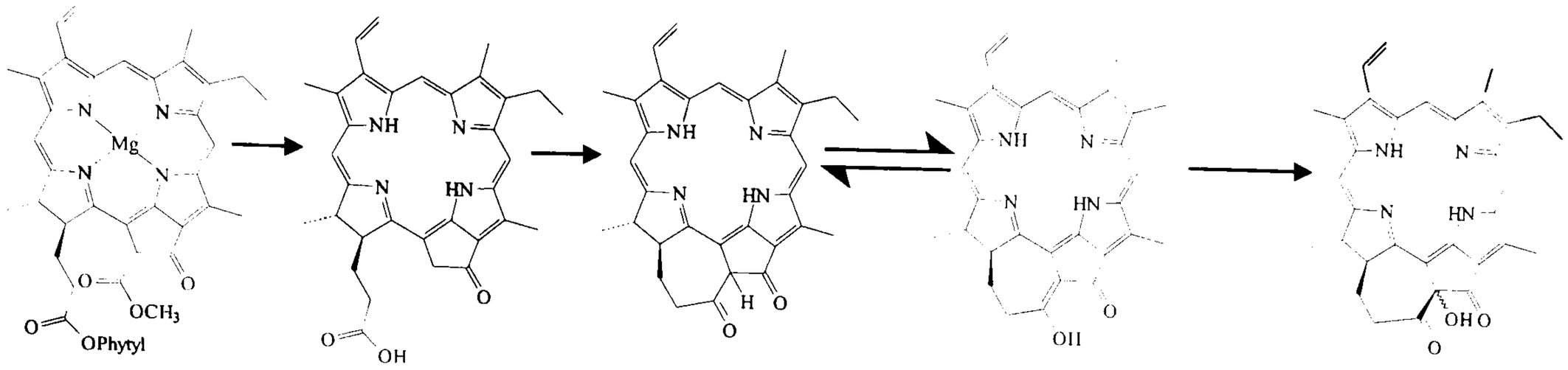


Figure 4-25. *Conversion of chl a to 13² -OH chlorophyllone a (adapted from Watanabe et al., 1993).*

The occurrence of chlorophyllone in sediments provides a link between chl *a* and a chlorin with a bicyclic ring system and two related porphyrins (fig. 4-26a-c; Keely, 1989; Prowse *et al.*, 1987) as well as an alkyl porphyrin with a seven-membered exocyclic ring (fig. 4-25c; Wolff *et al.*, 1983) all found in ancient sediments. Consequently, it seems likely that the occurrence of chlorophyllone in faecal pellets provides a rapid and efficient method of transport of this compound to the sediment prior to further alteration to produce bicycloalkanoporphyrins (Bi CAPs).

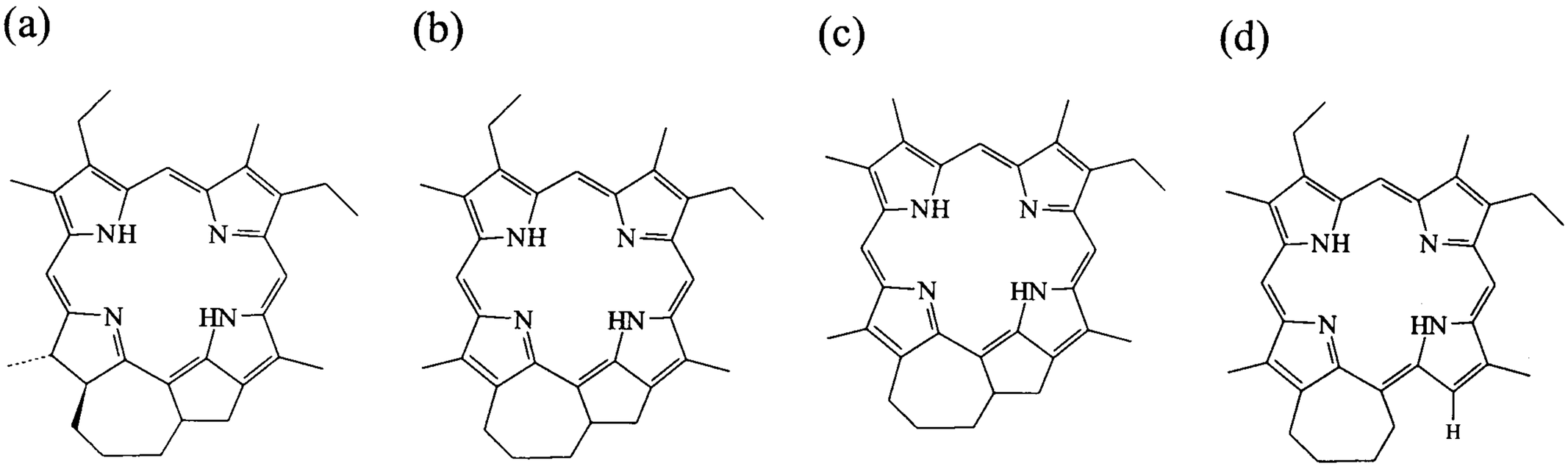


Figure 4-26. *Examples of sedimentary chlorins and porphyrins with seven membered exocyclic ring.*

The origin of the two novel 13²-oxopyrophaeophorbide *a* esters is unclear. Previously only the hydrolysed nucleus has been observed to occur naturally, being found in trace abundance in a clam and also suggested to be an antioxidant (Watanabe *et al.*, 1993). It was again thought to originate from the chlorophyll in diatoms (*cf.* chlorophyllone above). Watanabe *et al.* (1993) proposed that it is produced from chlorophyllone (**X**) by oxidative cleavage of the 7 membered ring; however, this would seem unlikely in this case given that in the more abundant component the esterifying alcohol (phytol) is still

present and does not occur in chlorophyllone (see fig. 4-27). It seems, therefore, that chlorophyllone is not a prerequisite for the production of 13^2-oxopyrophaeophorbide nucleus (fig. 4-27). It is likely therefore that the phytyl ester was present in trace abundance in the diatom culture but was not detected (as suggested earlier for chlorophyllone) and was converted to its SCE during grazing in the same way as "normal" SCEs are formed.

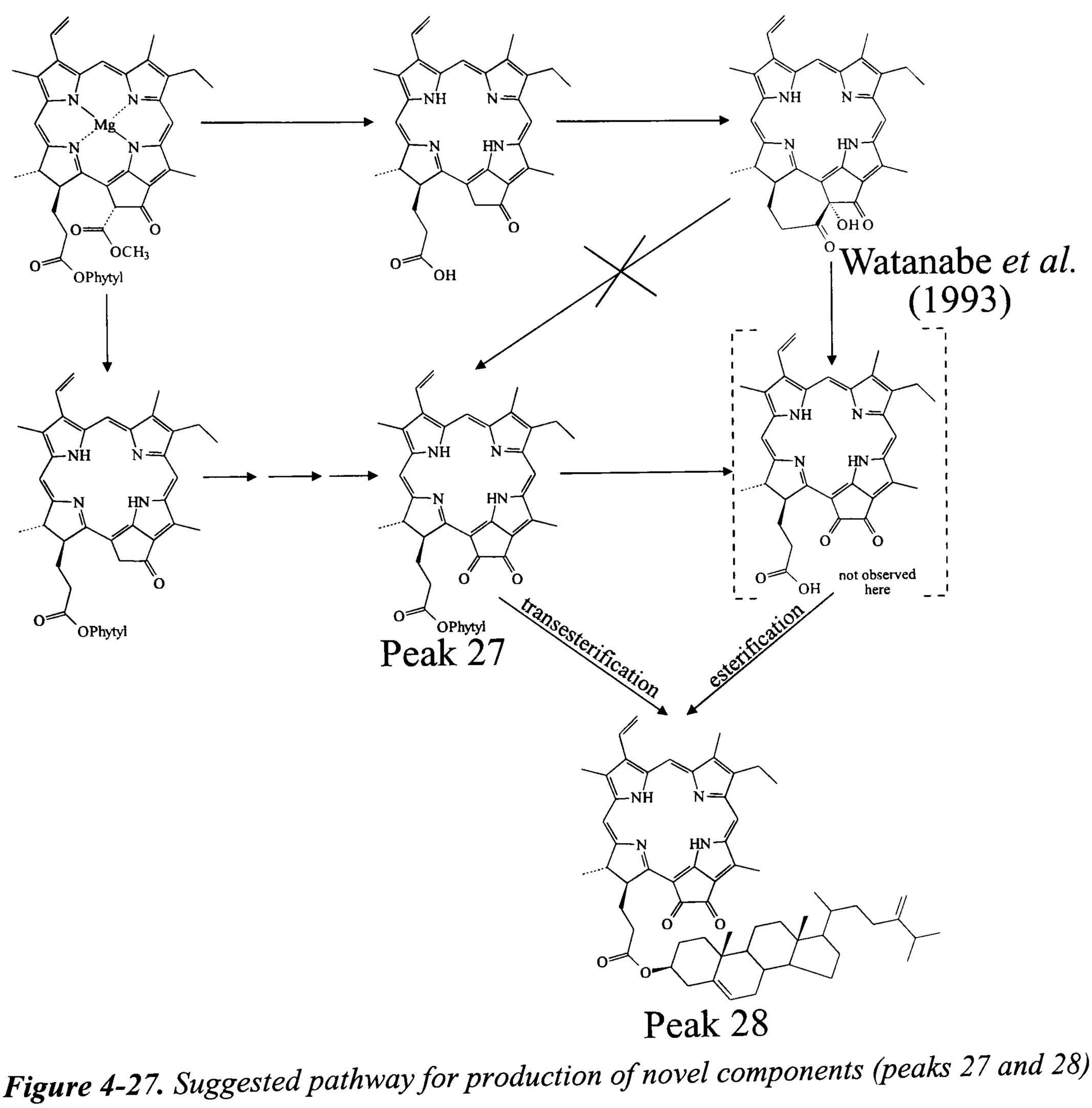


Figure 4-27. *Suggested pathway for production of novel components (peaks 27 and 28).*

4.5. Summary

The production of SCEs during grazing on a member of a third of the major algal divisions (the Bacillariophyta) has been confirmed with *C. helgolandicus* and the diatom *Thalassiosira weissflogii*. All of the available algal and animal sterols were incorporated into the SCE fraction; however, there was some alteration of the algal sterol distribution prior to esterification. The SCE sterol distribution remained constant over a period of faecal pellet ageing (30 d). This suggests that, allowing for any initial alteration as a result of dietary sterol assimilation (also applicable to free sterol distributions), prior to esterification, SCE sterols are more accurate indicators of phytoplankton community structure than are free sterols; the latter are susceptible to extensive alteration and degradation both during transport through the water column and during incorporation into sediments.

A number of known and novel compounds, some of which have been reported to be associated with diatoms, where they are thought to act as antioxidants, were also identified in the faecal pellets; this suggests that incorporation into pellet material provides an efficient transport mechanism for the removal of these components from the photic zone, resulting in their incorporation into sediments where further alterations lead to the production of sedimentary porphyrins of a variety of structural types.

Chapter 5

FEEDING EXPERIMENTS WITH DINOFLAGELLATES

5.1 INTRODUCTION

5.1.1. Background

Comparison of sedimentary free and SCE sterol distributions from a number of marine and lacustrine environments has highlighted consistent differences in the abundance of 4-methyl *vs*. 4-desmethyl sterols within the two fractions (King and Repeta, 1991, 1994; Eckardt *et al*., 1992; Pearce *et al*., 1998). For example, King and Repeta (1994) found that only 20% of the free sterols in a sediment from the Black Sea were 4-desmethyl sterols whilst 99% of the SCE sterols were 4-desmethyl components. The SCEs also showed a distribution corresponding favourably with the distribution in the total flux of free sterols to the sediment, which consisted of 98% 4-desmethyl sterols, with the balance in each case made up of 4-methyl sterols. This difference was ascribed to the greater resistance of the free 4-methyl sterols to biodegradation (e.g. Teece, 1994), indirectly suggesting that esterification to pyrophaeophorbide *a* would seem to protect 4-desmethyl sterols from selective degradation.

Sterols containing a 4-methyl substituent (e.g. 4α,23,24-trimethylcholest-22-en-3β-ol, dinosterol, **B10**) are of particular importance as they are largely derived from a single source, the Dinophyta (or dinoflagellates; see below) although they are not found in all species. They are commonly used as indicators of dinoflagellate input to sedimentary environments and the presence of the saturated hydrocarbon, dinosterane (**XXXXV**), thought to be derived from dinosterol, is commonly used as evidence of the presence of organic matter derived from dinoflagellates in sediments and oils and where present in high abundance is generally considered to indicate a marine origin whilst the related component 24-ethyl-4α-methylcholestane occurs in both marine and lacustrine environments (Summons *et al*., 1992 and references therein). It should be noted, however, that there have been a limited number of reports in which 4-methyl sterols have been identified in haptophytes from the class pavlovales (Conte *et al*., 1994, and references therein and see Chapter 3). A number of 4-methyl sterols, including dinosterol have also been identified in a marine diatom (*Navicula* sp., Volkman *et al*., 1993).

5.1.2. The Dinophyta

The Dinophyta or dinoflagellates are a diverse group of unicellular flagellates comprising *ca.* 130 genera and at least 1200 living species, half of which are photosynthetic, and the other half having heterotrophic or animal-like nutrition (Jeffrey and Vesk, 1997). They are mostly individual cells ranging in size from 5 to 2000 μm, although some are known to form chains. All dinoflagellates have a motile stage with two dissimilar flagella. They can occur as unarmoured or armoured forms covered in cellulose plates (thecal plates). Dinoflagellates are widely distributed in both the marine and lacustrine environment and form specific species assemblages in tropical, subtropical and temperate waters. Tropical species are usually large heterotrophic forms, some of which contain cyanobacterial symbionts. They are also often found in symbiosis with tropical invertebrates such as corals, clams and foraminifera. In temperate environments dinoflagellate species are usually smaller and can be either photosynthetic or heterotrophic. Dinoflagellate blooms (often referred to as red tides) mostly occur in coastal and inshore waters and often follow an initial diatom bloom (see Chapter 4).

Most photosynthetic dinoflagellates commonly contain chl *a* (**I**), chl c_2 (**IV**), β,β-carotene (**XXXV**), peridinin (**XXXXVI**), dinoxanthin (**XXXXVII**) and diadinoxanthin (**XXXVI**) (Jeffrey and Vesk, 1997).

5.1.3. Present Study

In order to compare the distribution of sterols in an algal substrate containing 4-methyl sterols with that in the resultant SCEs, two small scale feeding experiments were carried out in which *Calanus helgolandicus* was allowed to graze on two different dinoflagellates. The first species, *Prorocentrum micans* (size 30-40 μm), is a common and abundant species found in coastal areas world wide. This species of soft bodied or "naked" flagellate is known to be of high nutritional value to copepods and is readily assimilated during grazing (Harris, personal communication). The second species *Alexandrium tamarensis* (formerly *Gonyaulax tamarensis*; 28-40 μm) is a less common

species found in northern coastal areas which has a rigid shell or theca. Both experiments demonstrated the production of SCEs, however, as *A. tamarensis* was found to contain only two sterols and the SCEs produced from them were only trace components (see below) in the faecal pellets, the first alga, *P. micans*, which contained at least seven sterols, was chosen as a suitable substrate for a large scale (x3) experiment involving examination of both SCE and free sterol distributions and abundance in aged pellets as well as comparison of components in pellets which had been sterilised ($HgCl_2$) and then aged. An outline of the experiment, samples and analysis protocols is given in Figure 5-1.

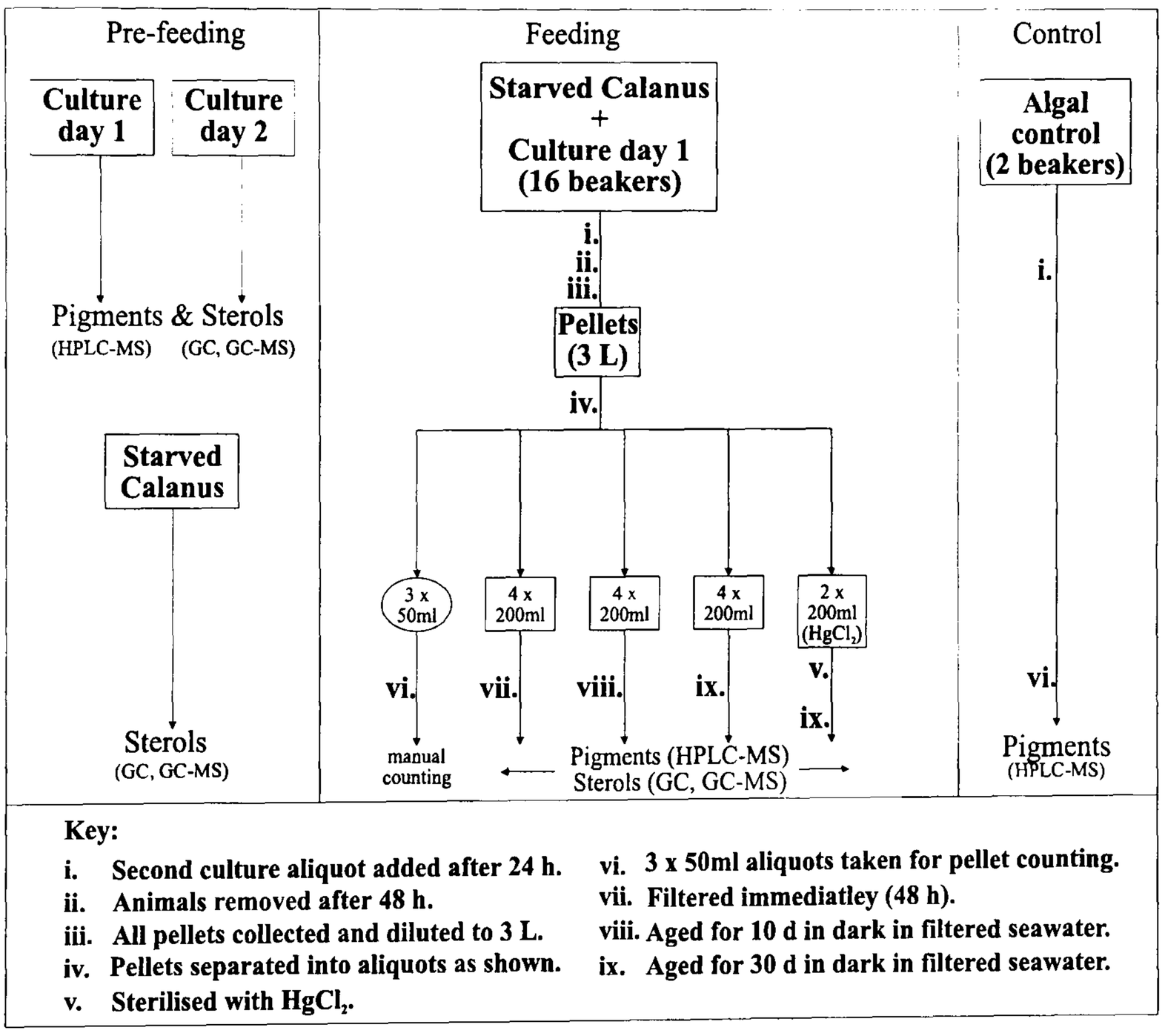


Figure 5-1. *Outline of samples and analysis protocols for large scale P. micans and C. helgolandicus feeding experiment.*

5.2. RESULTS: SMALL SCALE *P. MICANS*

5.2.1. Algal Culture

The only significant pigment components in the culture (fig. 5-2) are both C-13^2 epimers of phaeophytin *a* (8 and 8', **VIII**) and its mono-oxygenated allomer, hydroxyphaeophytin *a* (6 and 6', **XIII**). Also present are a number of carotenoids which were not investigated further. There is no indication of chl *a* (**I**) or its allomers which suggests that the culture may have reach stationary phase and started to undergo senescence.

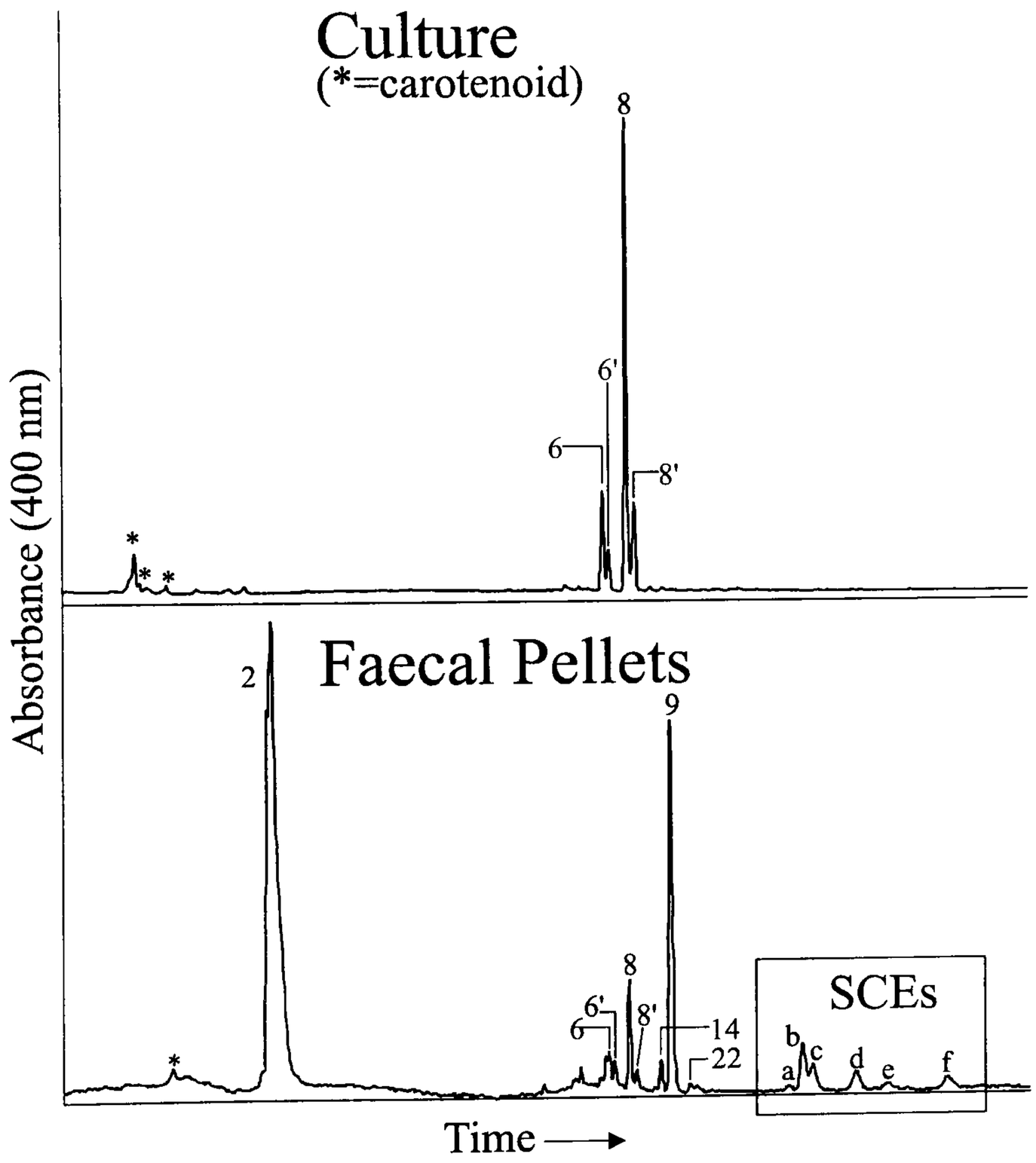


Figure 5-2. *HPLC chromatograms (400 nm) from copepod feeding on Prorocentrum micans (small scale experiment).*

5.2.2. Faecal Pellets

The pellet pigment distribution (fig. 5-2) is dominated by pyrophaeophorbide *a* (peak 2, **X**). Also present in significant abundance is pyrophaeophytin *a* (9, **XI**) and both C-13^2

epimers of phaeophytin *a* (8 and 8') and hydroxyphaeophytin *a* (6 and 6'). Purpurin-18-phytyl ester (14, **XIVa**) and peak 22 (*cf.* Chapters 3 and 4) were also present as minor components.

5.2.3. SCEs

The six peaks (a-f, fig. 5-2) in the SCE region have electronic spectra similar to that of pyrophaeophorbide *a* and were identified as SCEs in the usual way by mass chromatography (fig. 5-3). Mass spectra of individual peaks each showed the expected fragment at *m/z* 535. The SCE MH^+ values and assignments of the corresponding sterols are given in Table 5-1.

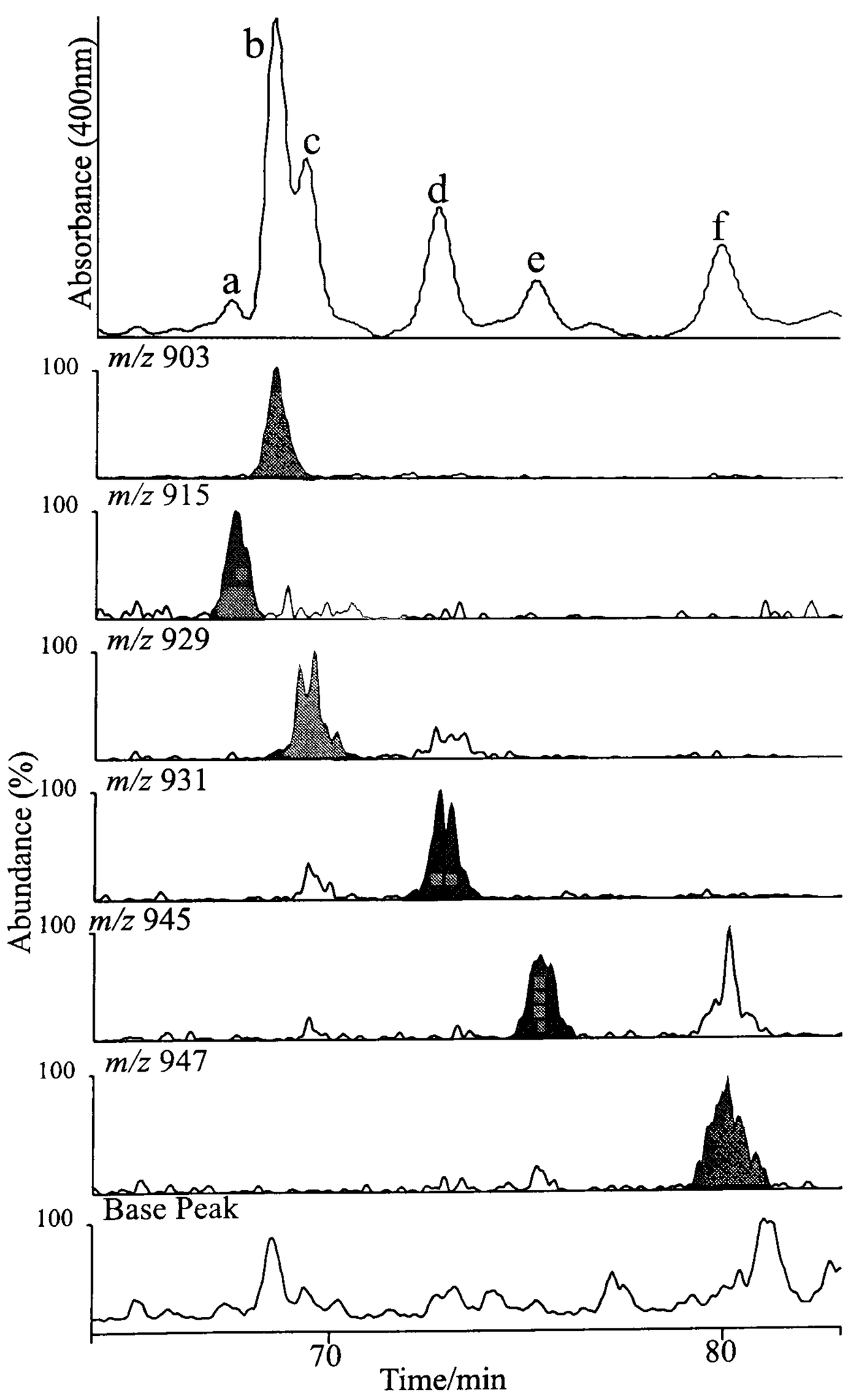


Figure 5-3. *Absorbance (400 nm) and mass chromatograms from faecal pellet SCE region (small scale P. micans experiment).*

Peak*	SCE MH^+	Esterified sterol
a	915	C_{28} 2 double bonds
b	903	C_{27} 1 double bond
c	929	C_{29} 2 double bonds
d	931	C_{29} 1 double bond
e	945	C_{30} 1 double bond
f	947	C_{30} 0 double bonds

Table 5-1. *SCE MH^+ and corresponding esterified sterol (* see fig. 5-3).*

5.2.4. Sterols

5.2.4.1. P. micans Culture

The distribution (fig. 5-4) contained a total of 7 sterols (see section 5.4. for mass spectra). The distribution is dominated by cholest-5-en-3β-ol (peak 2, **A1**), with other 4-desmethyl components being 24-methylcholesta-5,22-dien-3β-ol (peak 5, **A6**), 23,24-dimethylcholesta-5,22-dien-3β-ol (peak 7, **A10**) and 24-ethylcholest-5-en-3β-ol (peak 10, **A8**). Also present were three 4-methyl sterols: 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol; peak 13, **B11**), 4α,23,24-trimethylcholest-22-en-3β-ol (dinosterol; peak 12, **B10**) and 4α,24-dimethylcholest-22-en-3β-ol (peak 9, **B6**).

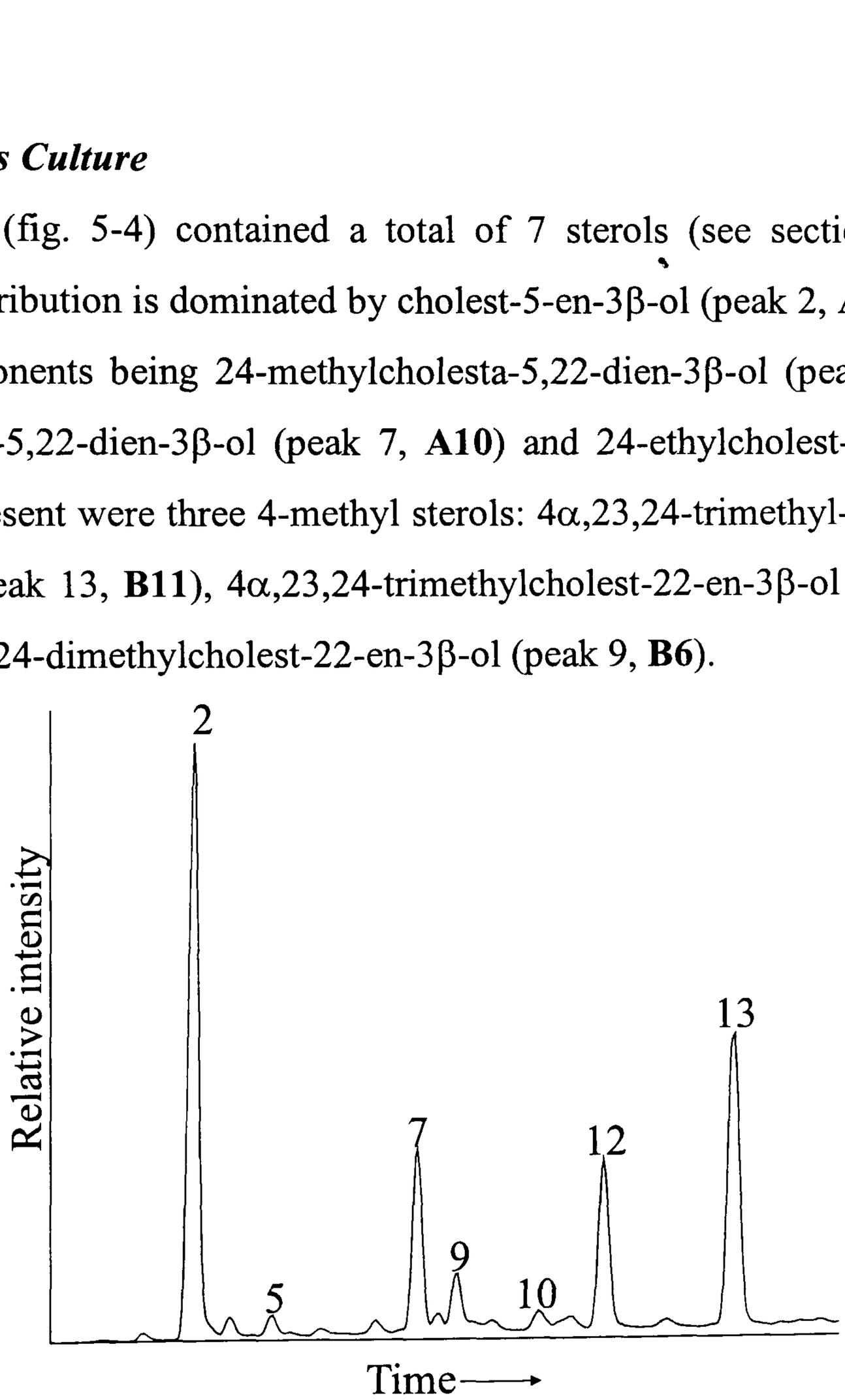


Figure 5-4. *Partial RIC trace of free sterols (as TMSi ethers) in algal culture (unlabelled peaks are non-sterol).*

5.2.4.2. Comparison of SCE and Culture Sterols

A total of seven sterols were detected in the culture, with molecular weights corresponding to those expected from the SCE mass chromatograms (fig. 5-3). There are, however, two sterols with masses corresponding to that of the SCE with $MH^+ = 931$ (24-ethylcholest-5-en-3β-ol [peak 10, **A8**] and 4α,24-dimethylcholest-22-en-3β-ol [peak g, **B6**]). As a standard of 4α,24-dimethylcholest-22-en-3β-yl pyrophaeophorbide *a* was not available for determination of its retention time and there was insufficient faecal material to attempt to isolate the SCE sterols by hydrolysis, this SCE peak is assumed to be composed of the co-eluting esters of each of the mono-unsaturated C_{29} sterols; the abundance of this SCE peak is therefore compared with the total available C_{29} mono-unsaturated sterol (fig. 5-5), although unfortunately this means that it is compared with contributions from both a 4-methyl and 4-desmethyl sterol. The other SCE MH^+ values correspond to a single available sterol and are assigned accordingly (Table 5-2).

SCE MH^+	Esterified sterol	Sterol structure
903	cholest-5-en-3β-ol	**A1**
915	24-methylcholesta-5,22-dien-3β-ol	**A6**
929	24-ethylcholesta-5,22-dien-3β-ol	**A9**
931	24-ethylcholest-5-en-3β-ol	**A8**
	4α,24-dimethylcholest-22-en-3β-ol	**B6**
945	4α,23,24-trimethylcholest-22-en-3β-ol	**B10**
947	4α,23,24-trimethyl-5α-cholestan-3β-ol	**B11**

Table 5-2. *SCE MH^+ and corresponding sterol.*

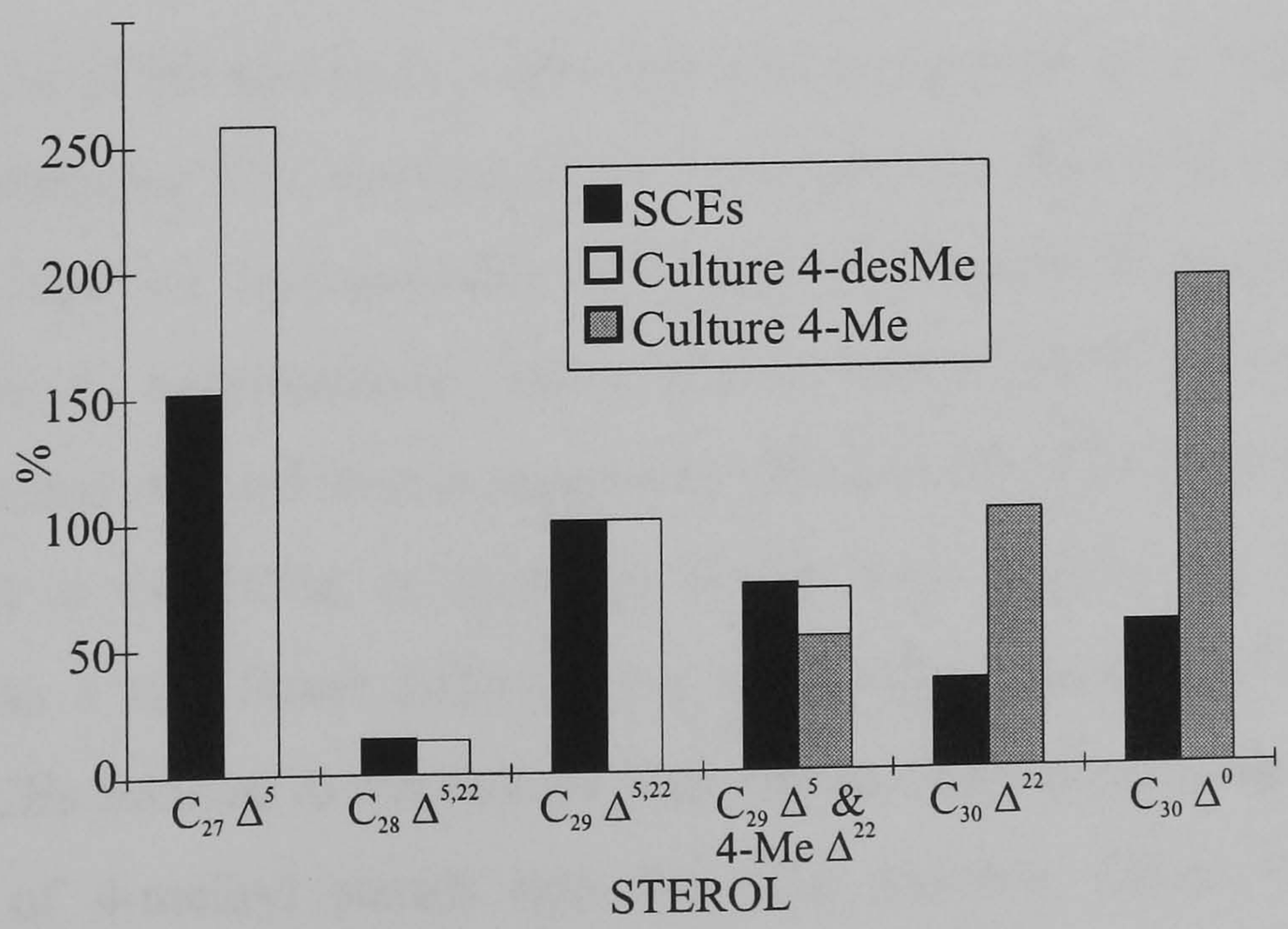


Figure 5-5. *Relative % of SCE and algal sterols.*

There was no evidence of the two diunsaturated C_{27} animal sterols (SCE MH^+=901) in the SCE fraction, so all SCE sterols are assumed to be derived from the alga in this case although there is probably a minor contribution to the cholest-5-en-3β-ol SCE (MH^+=903) from the animal. There is a significant reduction in the proportion of cholest-5-en-3β-ol in the SCEs relative to that in the alga, suggesting significant uptake of cholest-5-en-3β-ol (**A1**) by the animal. The good abundance correlation between the other 4-desmethyl sterols (24-methylcholesta-5,22-dien-3β-ol and 24-ethylcholesta-5,22-dien-3β-ol) and their corresponding SCEs is thought to be due to the presence of a significant abundance of cholest-5-en-3β-ol in the alga, eliminating the need in this case for the assimilation of the C-24 alkylated sterols for the production of cholesterol (unlike other algae used as substrates, Chapters 2-4). There is also a significant reduction in the abundance of the two major 4-methyl sterols, dinosterol and dinostanol. The situation with the two C_{29} mono-unsaturated sterols is unclear as there is little difference between the two total abundances; this suggests that if the 4-methyl discrimination effect observed here for the two major 4-methyl sterols is taking place, then there appears to have been enhanced incorporation of the desmethyl sterol into the SCEs, unlike that observed for the other desmethyl sterols (excluding cholest-5-en-3β-ol) as this component (peak 10 in fig. 5-4) represents only *ca.* 20% of the total available sterol contributing to this SCE peak.

5.2.5. Summary

The production of SCEs has been confirmed with a member of a fourth major algal division, the Dinophyta. The dinoflagellate *P. micans* was found to contain a total of seven sterols at least six (and probably seven) of which were incorporated into SCEs during grazing by *C. helgolandicus*. There was no indication of the two common C_{27} diunsaturated animal-derived sterols (cholesta-5,22-dien-3β-ol [**A3**] and cholesta-5,24-dien-3β-ol [**A2**]) in the SCEs, as presumably they were present but below detection limits. There was a significant difference in the relative abundance of the 4-methyl sterols in the SCEs relative to the culture free sterols, with an apparent discrimination against uptake of 4-methyl sterols into the SCE fraction. Other observations are discussed in Section 5.5.

5.3. RESULTS: *A. TAMARENSIS* EXPERIMENT

5.3.1. Algal Culture

The culture pigment distribution (fig. 5-6) is dominated by both C-13[2] epimers of phaeophytin *a* (8 and 8') and hydroxyphaeophytin *a* (6 and 6') suggesting again that this culture had started to undergo senescence prior to feeding (*cf. P. micans* above).

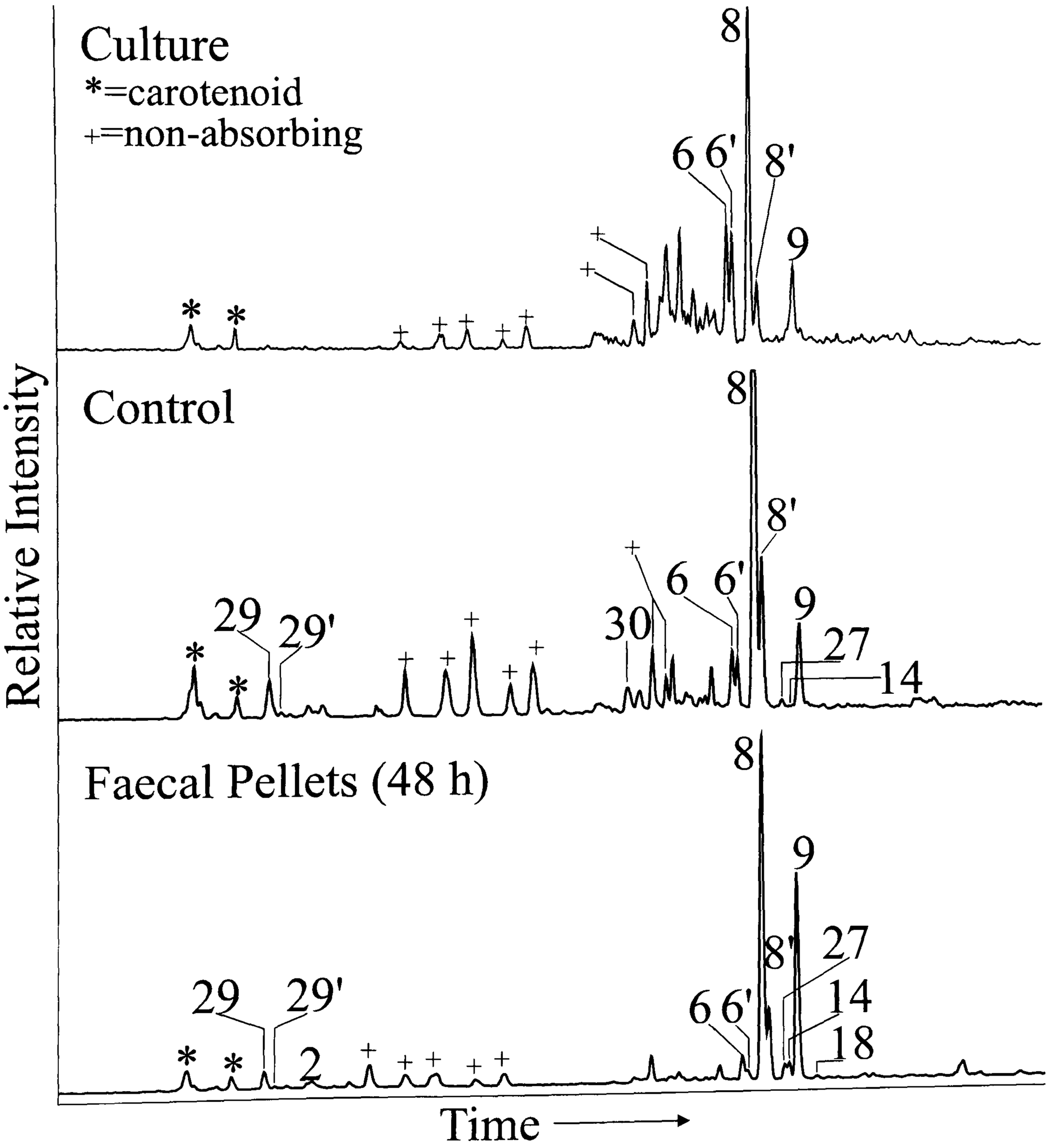


Figure 5-6. *LC-MS base peak traces from copepod feeding on A. tamarensis.*

Surprisingly, pyrophaeophytin *a* (9, **XI**) is also present, providing further evidence that the culture had reached stationary phase and had started to undergo senescence prior to feeding. There were a number of other chl related components (unlabeled peaks) which could not be identified due to a hard disk failure leading to loss of the electronic data; a number of non-absorbing components were also present as well as a group of carotenoids which were not investigated further.

5.3.2. Algal Control

The pigment distribution (fig. 5-6) is similar to that of the culture, again dominated by phaeophytin *a* (8 and 8'), hydroxyphaeophytin *a* (6 an 6') and pyrophaeophytin *a* (9). There were again also a similar number of non-absorbing components and carotenoids. There were, however, a number of other chl *a* transformation products present including both C-13^2 epimers of hydroxychlorophyllone (**XVI**; peaks 29 and 29'; e.g. Sakata *et al.*, 1990, Watanabe *et al.*, 1993; see also Chapter 4). The mass spectra of the two epimers are shown in Figure 5-7. The ion at *m/z* 553 in Figure 5-7a is thought to result from co-elution with a carotenoid as it is not present in the spectrum of the second epimer.

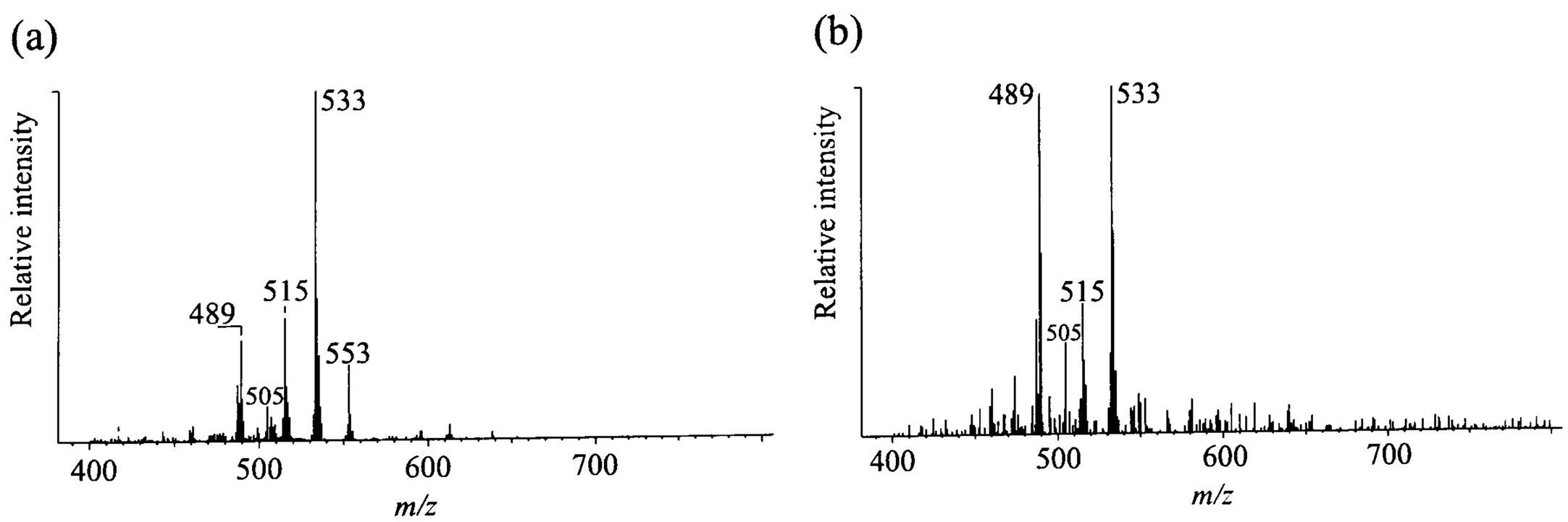


Figure 5-7. *Mass spectra of (a) peak 29 (13^2-hydroxychlorophyllone) and (b) peak 29' (13^2-epi-hydroxychlorophyllone).*

The mass spectrum of peak 30 (fig. 5-8a) consists of a single ion (MH^+) at *m/z* 517 consistent with 13^2,17^3-cyclophaeophorbide *a* enol (**XXXXIV**) (e.g. Ocampo *et al.*, 1999a). This assignment is also indicated by comparison of the electronic spectrum (not shown) with that given by Ocampo *et al.* (1999a; $\lambda_{max.}$ 360, 416, 628, 686 nm). Also

present is peak 27, previously observed in the faecal pellets from the large scale *T. weiss* experiment (Chapter 4) and assigned as 13^2-oxopyrophaeophytin *a* (fig. 5-8b). Purpurin-18-phytyl ester is also present (peak 14, **XIVa**).

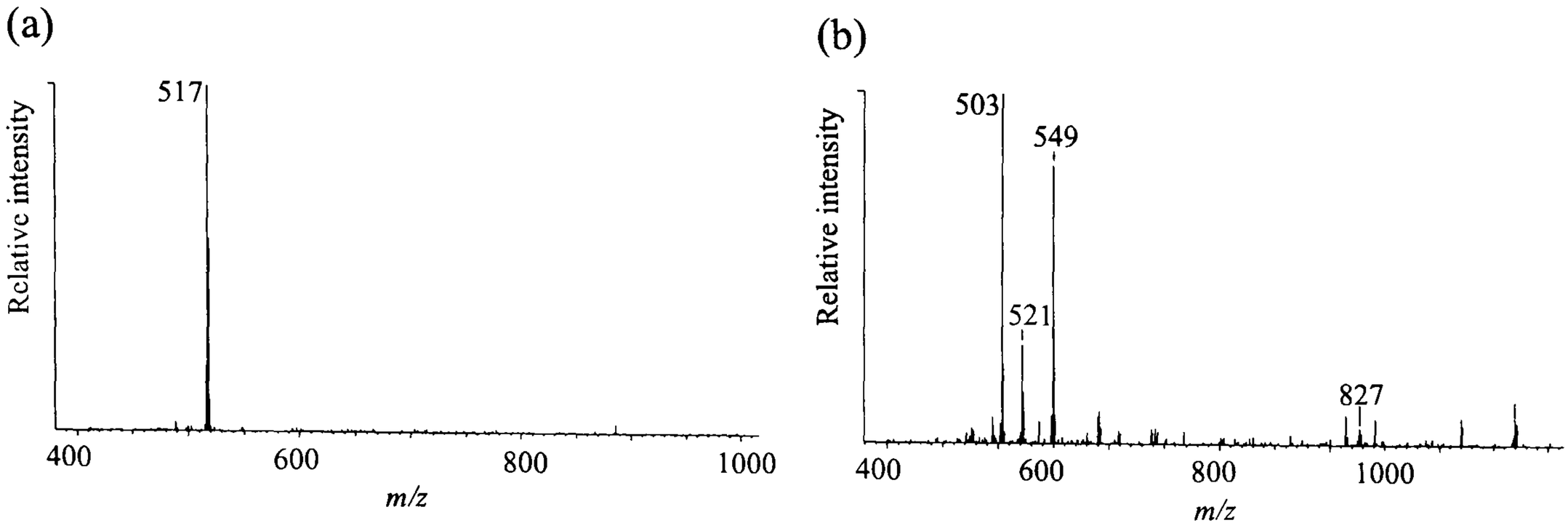


Figure 5-8. *Mass spectra of (a) Peak 30 (13^2,17^3-cyclophaeophorbide a enol) and (b) Peak 27 (13^2-oxopyrophaeophytin a).*

5.3.3. Faecal Pellets

The pigment distribution (fig. 5-6) is similar to that of the algal control and is again dominated by both C-13^2 epimers of phaeophytin *a* (8 and 8') and with an increased proportion of pyrophaeophytin *a* (9) relative to phaeophytin *a*, indicating production of this component during grazing (*cf.* Chapters 2, 3 and 4). There is no indication of peak 30 (13^2,17^3-cyclophaeophorbide *a* enol); however, both C-13^2 epimers of chlorophyllone (29 and 29', **XVI**), 13^2-oxopyrophaeophytin *a* (27) and purpurin-18-phytyl ester (14, **XIVa**) are again present. The latter is present in relatively high abundance as indicated by the MH^+ at *m/z* 843 (fig. 5-9a) in contrast to its abundance in previous pellet samples (*cf.* Chapter 2, fig. 2-29). Peak 18, eluting immediately after pyrophaeophytin *a*, has a similar mass spectrum (fig. 5-9b) to that of the same peak in Chapter 3, which was present in the culture and control of *P. carterae*, but in that case was absent from the faecal pellets but was present in the faecal pellets from the small scale prasinophyte experiment (fig. 2-9b in Chapter 2). This non-polar component appears to contain a phytyl substituent but could not be identified further.

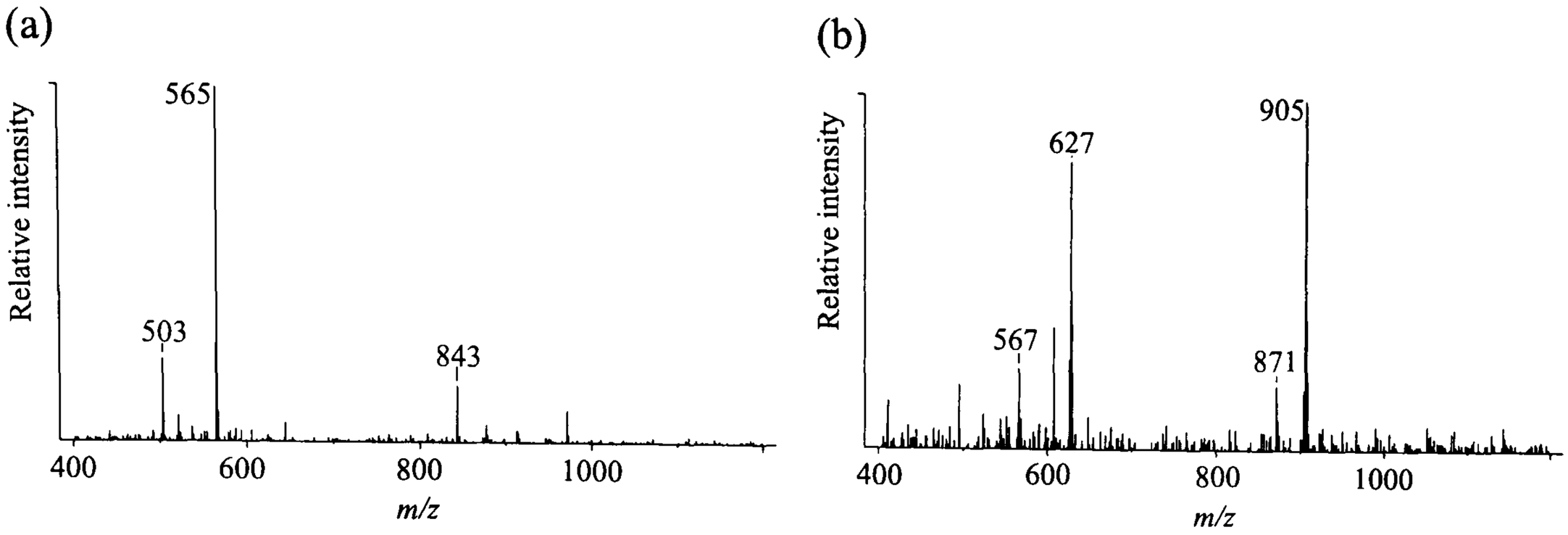


Figure 5-9. *Mass spectra of (a) peak 14 (purpurin-18-phytyl ester) and (b) peak 18.*

Mass chromatography (fig 5-10) revealed two SCE peaks (a and b). Mass spectra (fig. 5-11) showed the expected fragment ion at *m/z* 535, although the presence of a number of other ions reflects their trace abundance. Peak a with MH^+ at *m/z* 903 (fig. 5-11a) indicates the esterifying sterol is a mono unsaturated C_{27} sterol and peak b with MH^+ at *m/z* 945 (fig. 5-11b) indicates the esterifying sterol is a monounsaturated C_{30} sterol. The pellets aged for 30 d showed essentially the same pigment distribution and again two SCEs in trace abundance.

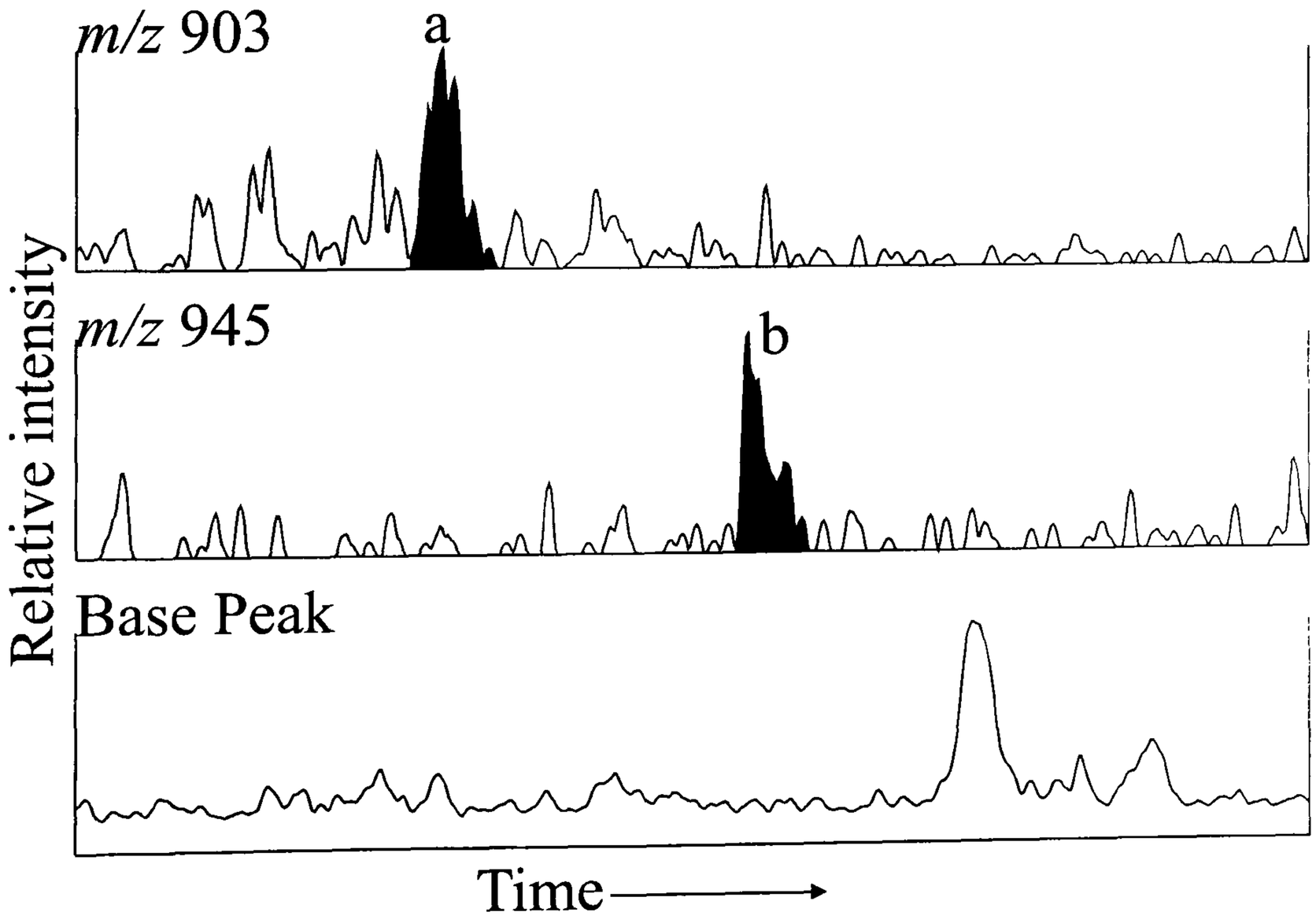


Figure 5-10. *Mass chromatograms of faecal pellet SCE region.*

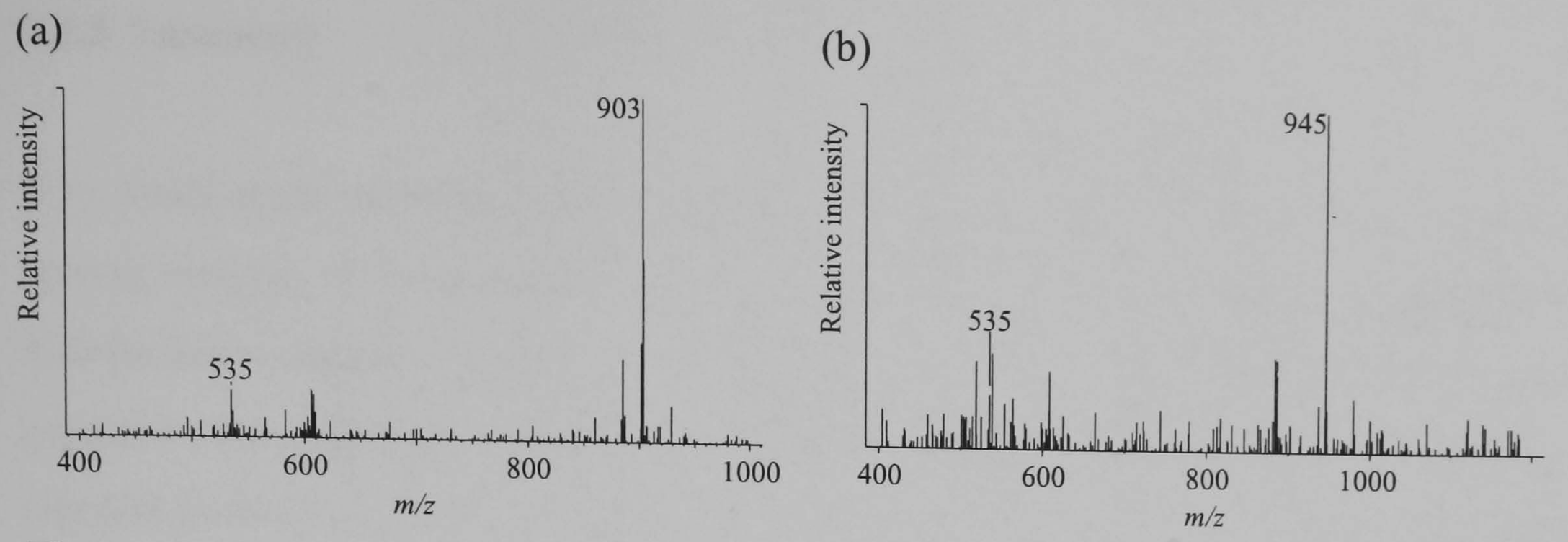


Figure 5-11. *Mass spectrum of (a) SCE peak* ***a*** *(MH^+=903) and (b) SCE peak* ***b*** *(MH^+=945).*

5.3.4. Sterols

The culture contained only two sterols (cholest-5-en-3β-ol [**A1**] and 4α,23,24-trimethylcholest-22-en-3β-ol [**B10**]), with masses directly matching those predicted by the SCE results. Comparison of the relative abundance of the free and SCE sterol (based on MH^+ peak areas; fig. 5-10) again (*cf. P.micans*) shows significant discrimination against the uptake of the 4-methyl sterol relative to that of the 4-desmethyl sterol (fig 5-12). It should be noted, however, that there is probably a small contribution of cholest-5-en-3β-ol from the animal although this is again thought to be negligible (*cf. P. micans*) as there is no indication of the two diunsaturated C_{27} sterols commonly found in the copepods and previously found to be incorporated into the SCEs (*cf.* Chapters 2-4).

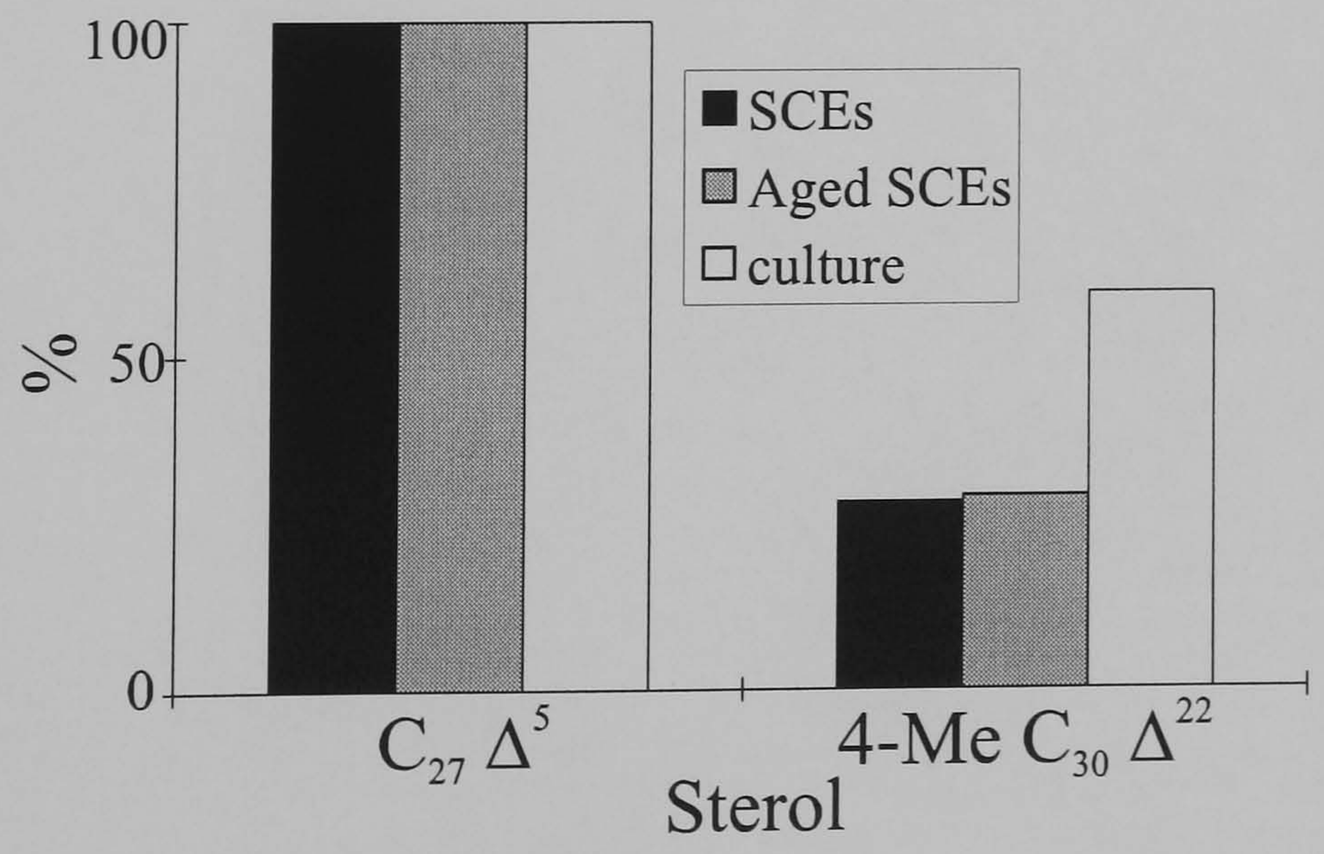


Figure 5-12. *Comparison of SCE and culture free sterol relative abundance.*

5.3.5. Summary

This small scale experiment has shown the production of SCEs during grazing on a second member of the Dinophyta algal division (*A. tamarensis*) and has confirmed the discrimination against 4-methyl sterols in forming SCEs. As the SCEs produced were present in trace abundance and there was a limited number of sterols present in the alga (cholest-5-en-3β-ol [**A1**] and 4α,23,34-trimethylcholest-22-en-3β-ol [**B10**]) this alga was thought to be an unsuitable substrate for a large scale experiment involving ageing of pellet aliquots for investigation of SCE and free sterol distribution and abundance during ageing. The *A. tamarensis* results do suggest, however, that no discrimination occurs during ageing which would alter the SCE sterol distribution.

5.4. RESULTS: LARGE SCALE *P. MICANS*

5.4.1. Algal Culture

The pigment distribution (fig. 5-13) is dominated by both C-13^2 epimers of phaeophytin *a* (peaks 8 and 8', **VIII**) and their mono oxygenated allomers hydroxyphaeophytin *a* (peaks 6 and 6', **XIII**) as observed in the small scale experiment (fig. 5-2).

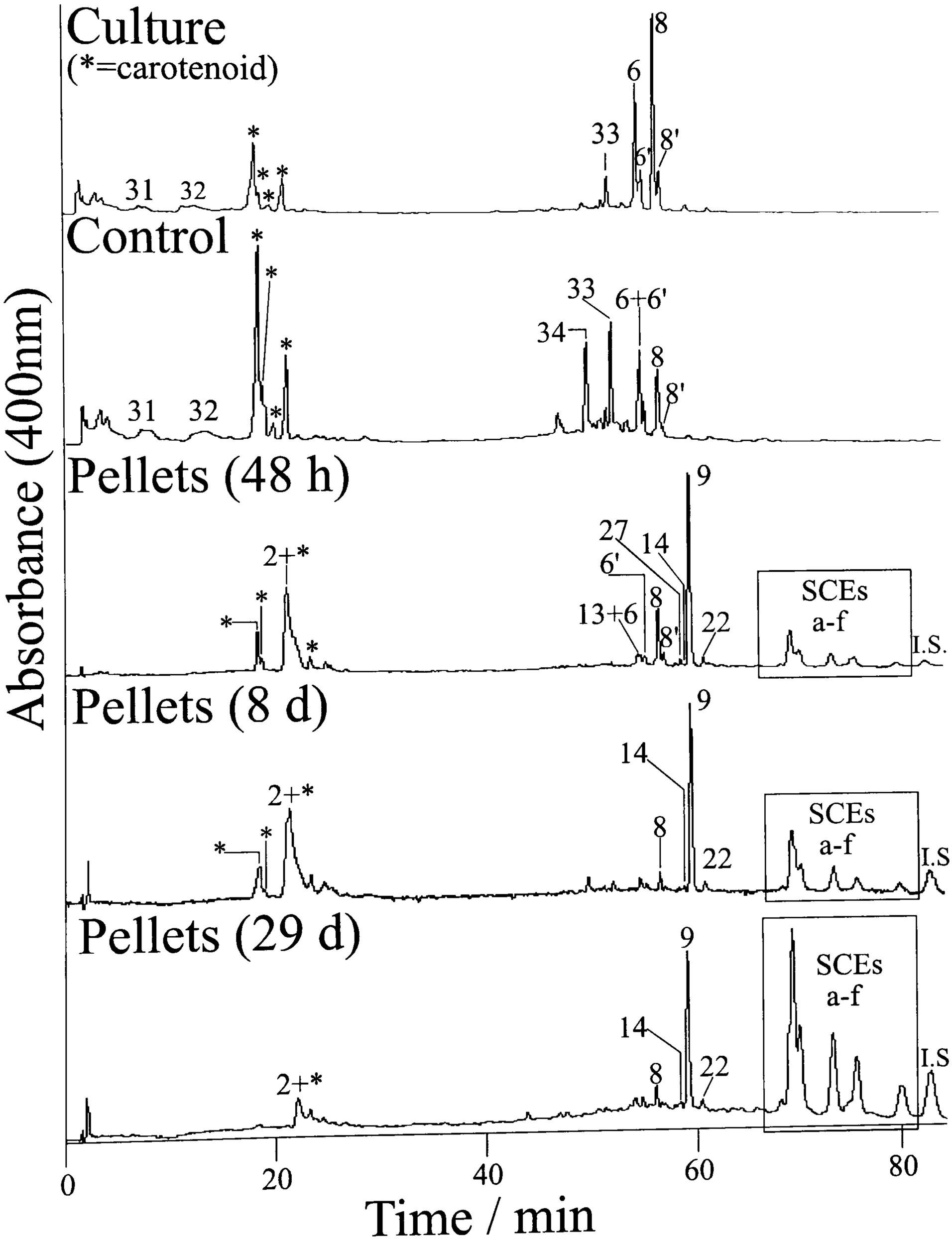


Figure 5-13. *HPLC chromatograms (400 nm) from large scale P. micans feeding experiment.*

Also present are two chl *c*-like components (peaks 31 and 32), but they were only detected from the electronic data and so could not be identified further. The electronic spectrum of peak 33 ($\lambda_{max.}$ 431, 587, 627 and 666 nm) is similar in structure to that of the unassigned peak 16 in Chapter 3 and the retention time is also similar; however, comparison of the mass spectra (fig. 5-14; and see fig. 3-3 in Chapter 3) suggests that the two components are not related. It was not possible to derive the structure of this component from the mass spectrum although it does appear to contain one or more additional oxygens indicating structural analogies with the assignment of peak 16 in Chapter 3 as a oxygenated chl *a*-like component but also with appreciable differences. A number of carotenoids were also observed but were not investigated further.

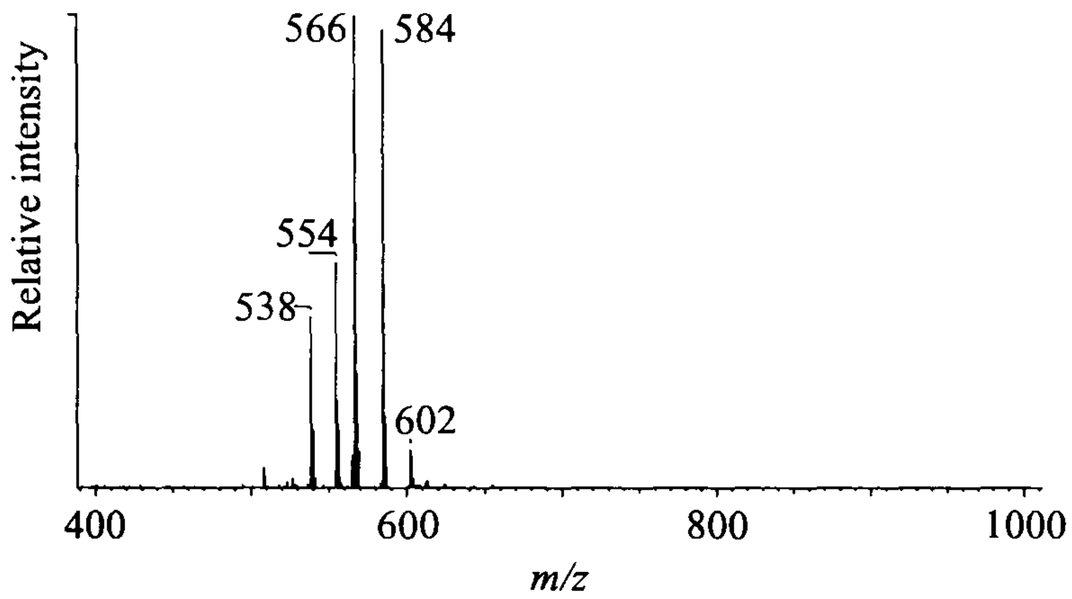


Figure 5-14. *Mass spectrum of peak 33.*

5.4.2. Algal Control

The pigment distribution (fig. 5-13) is qualitatively similar to that of the culture except for the addition of peak 34. The electronic spectrum of this component is similar to that of chlorophyll *a* ($\lambda_{max.}$ 428, 528, 572, 615 and 662 nm; *cf.* Chapter 2), but the mass spectrum (fig. 5-15) is different. The ions at *m/z* 631 and 613 suggest the presence of the chl *a* mono-oxygenated allomer (*cf.* Peak 12; Chapter 2). The ion at *m/z* 522 is thought to be derived from a non-absorbing co-eluting component as it was observed in other samples where there was no corresponding peak in the 400 nm chromatogram. Overall there is a significant reduction in the abundance of all of the chl related components relative to the carotenoids.

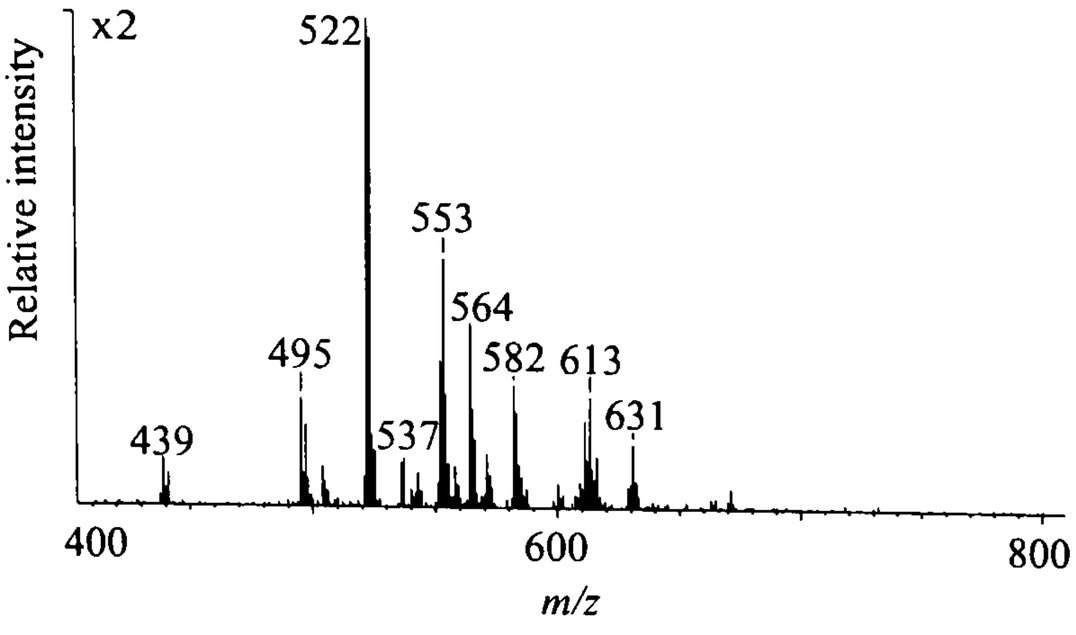


Figure 5-15. *Mass spectrum of peak 34.*

5.4.3. Faecal Pellets (0 d)

The distribution (fig. 5-13) in the fresh pellets is dominated by pyrophaeophytin *a* (peak 9, **XI**) along with both C-13^2 epimers of phaeophytin *a* (8 and 8') and hydroxyphaeophytin *a* (6 and 6') as well as 15^1-hydroxyphaeophytin *a* lactone (13, **XXXIII**). Also present, co-eluting with a group of carotenoids, is pyrophaeophorbide *a* (2, **X**). Other minor components are purpurin-18-phytyl ester (14, **XIVa**), the unknown peak 22 (see also Chapter 3) and peak 27 (13^2-oxopyrophaeophytin *a*; see Chapter 4) which were also present in the small scale experiment. A suite of 6 peaks (a-f) were also observed in the SCE region (*cf.* small scale experiment and see below).

5.4.4. Faecal Pellets (8 d)

After ageing the distribution is again dominated by pyrophaeophytin *a* (9) and pyrophaeophorbide *a* (2), again co-eluting with a group of carotenoids. Phaeophytin *a* (8 and 8'), hydroxyphaeophytin *a* (6 and 6'), purpurin-18-phytyl ester (14) and peak 22 are also present in trace abundance. The SCEs show greater abundance relative to pyrophaeophytin *a* than in the fresh pellets.

5.4.5. Faecal Pellets (29 d)

After ageing for 29 d the distribution is clearly dominated by the SCEs, with the proportion of pyrophaeophytin *a* significantly reduced. Pyrophaeophorbide *a* (2), a

reduced number of carotenoids, phaeophytin *a* (8 and 8'), purpurin-18-phytyl ester (14) and peak 22 are present as minor components.

5.4.6. Sterilised Faecal Pellets (29 d)

The sterilised pellet pigment distribution (fig. 5-16) is similar to that of the fresh pellets except for the absence of pyrophaeophorbide *a* (2). The absence of this minor component is difficult to explain. It is perhaps significant, however, that it was the only acidic pigment and hence may have formed the salt. Alternatively there may have been a chelating effect of the Hg^{2+} ion (from $HgCl_2$ used to sterilise the pellets) producing a complex (*cf.* Quirke and Maxwell, 1980) not observed under the HPLC conditions used (interestingly all the other pigments present are esterified components so it is also possible that the Hg^{2+} salt was formed). Again there was no evidence of the chls *c* but the other common products (i.e. phaeophytin *a* [8 and 8'] and pyrophaeophytin *a* [9]) as well as the six SCE peaks (a-f) were observed in abundances comparable with the fresh pellet sample. A number of other chl-like components were apparent from the electronic data but not the mass spectral data.

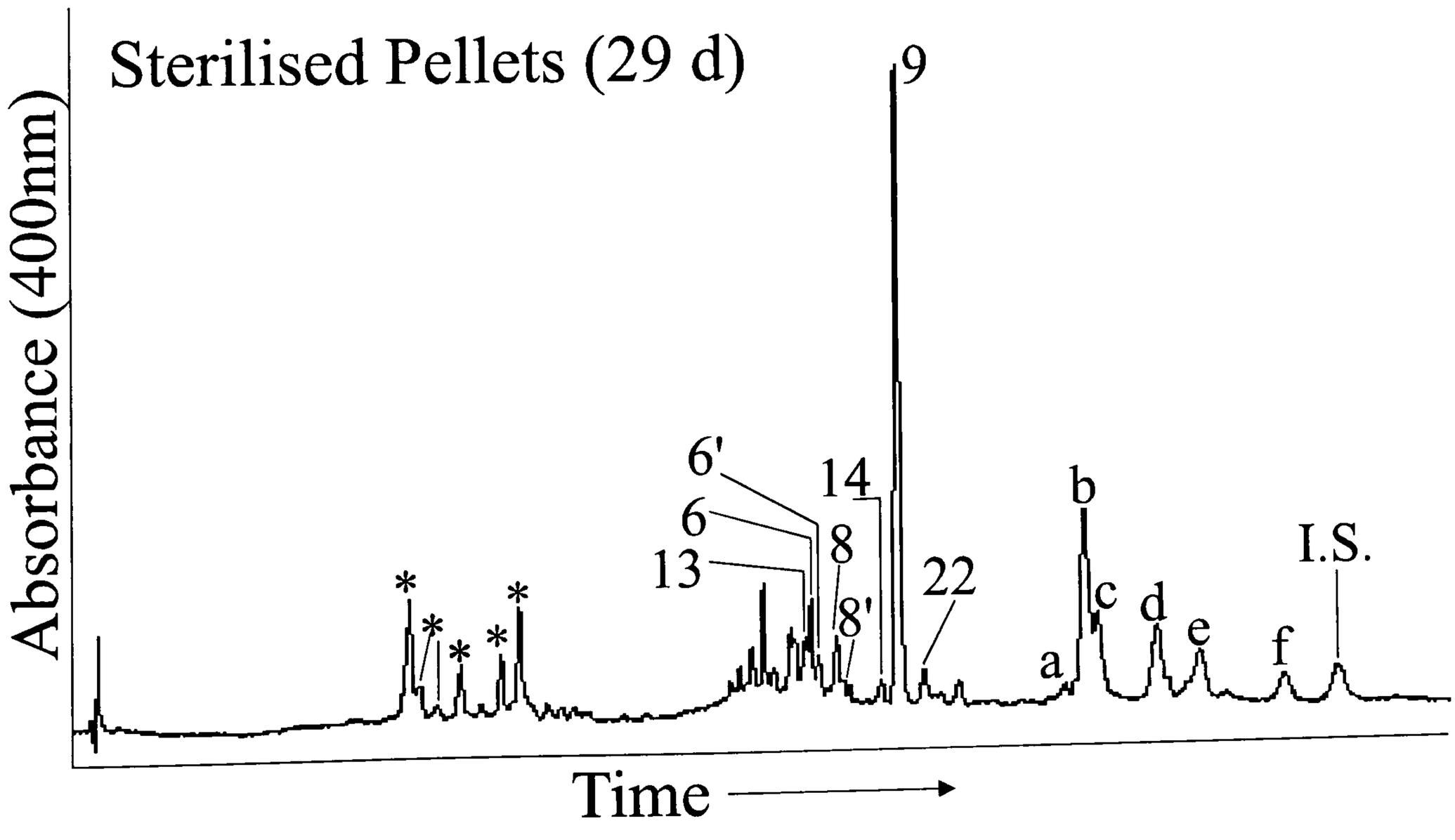


Figure 5-16. *HPLC chromatogram (400 nm) of sterilised ($HgCl_2$) faecal pellets.*

5.4.7. SCEs

Mass chromatography (fig. 5-17) confirmed the presence of a total of 7 MH^+ ions consistent with C_{27}-C_{30} mono and diunsaturated or fully saturated sterols esterified to pyrophaeophorbide *a* (**X**). SCE MH^+ and corresponding sterols are given in Table 5-3.

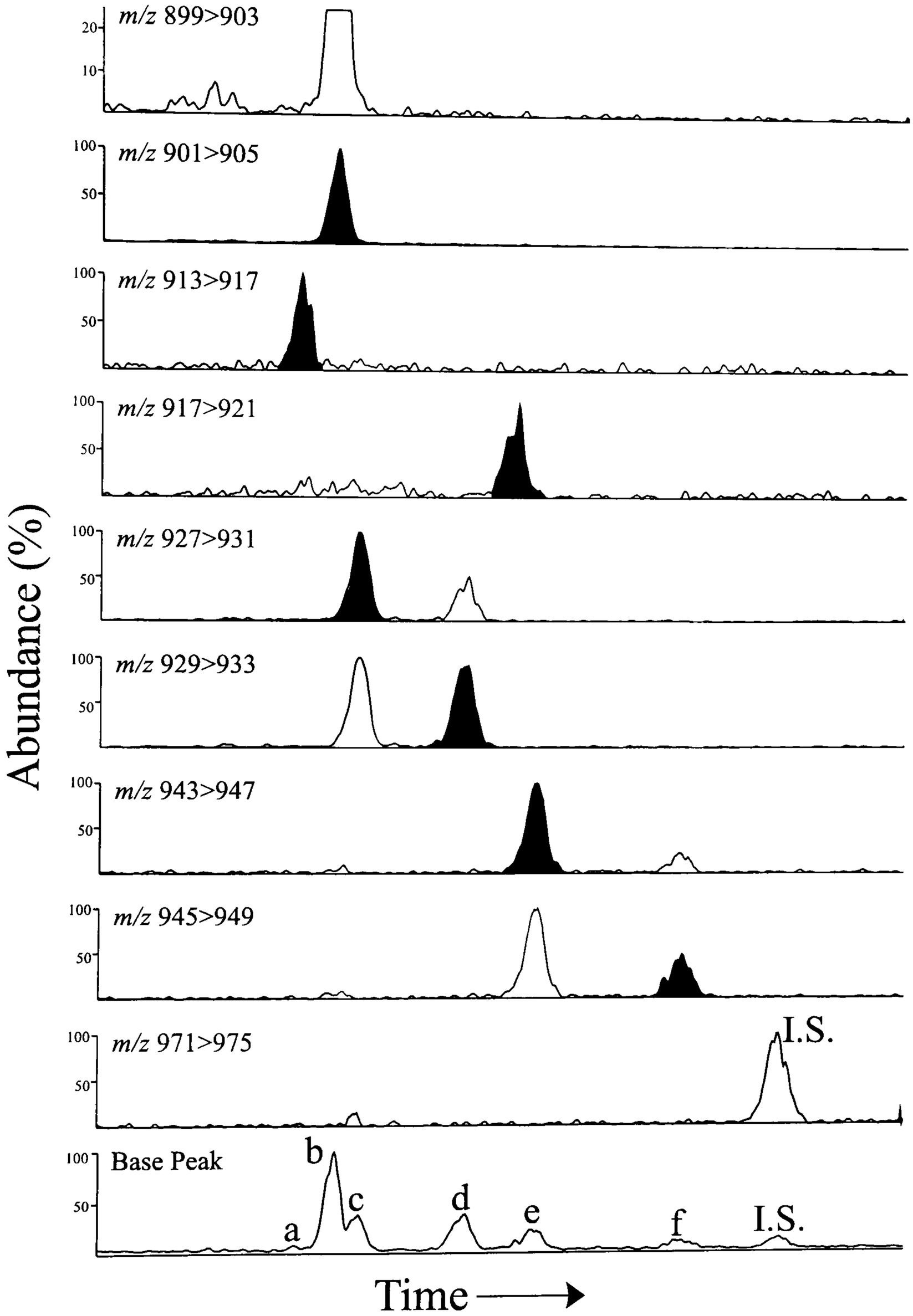


Figure 5-17. *Faecal Pellet SCE region mass chromatograms (shaded peaks are SCE MH^+).*

Peak	SCE MH^+	Corresponding sterol(s)
b	903	C_{27} 1 double bond
a	915	C_{28} 2 double bonds
e	919	C_{28} 0 double bonds
c	929	C_{29} 2 double bonds
d	931	C_{29} 1 double bond
e	945	C_{30} 1 double bond
f	947	C_{30} 0 double bonds

Table 5-3. *SCE MH^+ and corresponding esterified sterol.*

Mass spectra (figs. 5-18 to 5-23) of the SCE peaks (a-f, fig. 5-13) all showed the expected fragment at *m/z* 535. Peaks a-d and f each consisted of a single SCE, peak e consisting of 2 SCEs with MH^+ 919 eluting just prior to MH^+ 945.

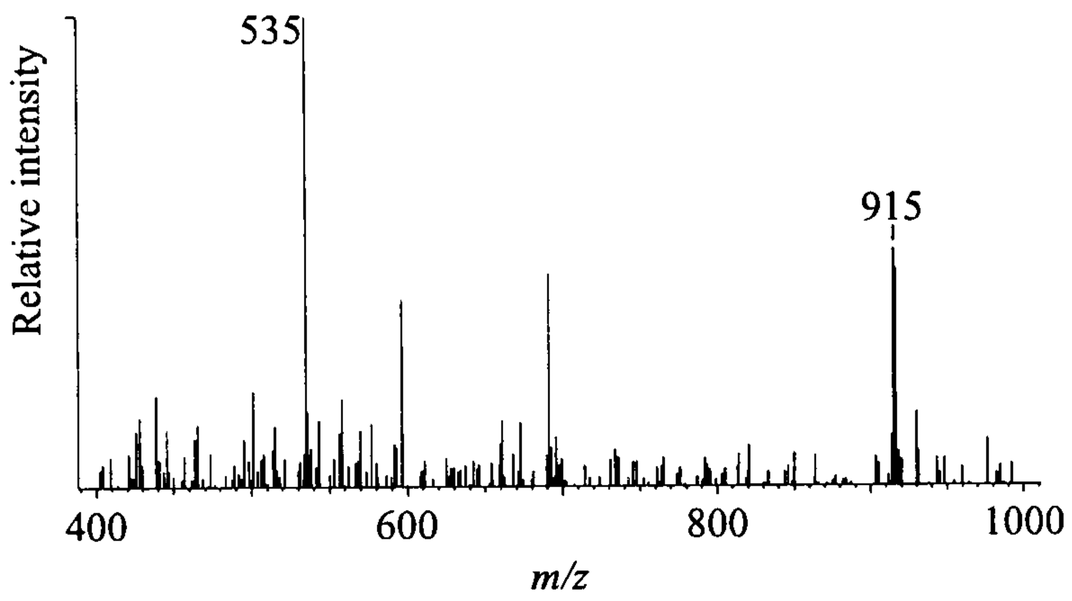


Figure 5-18. *Mass spectrum of SCE peak a (MH^+=915).*

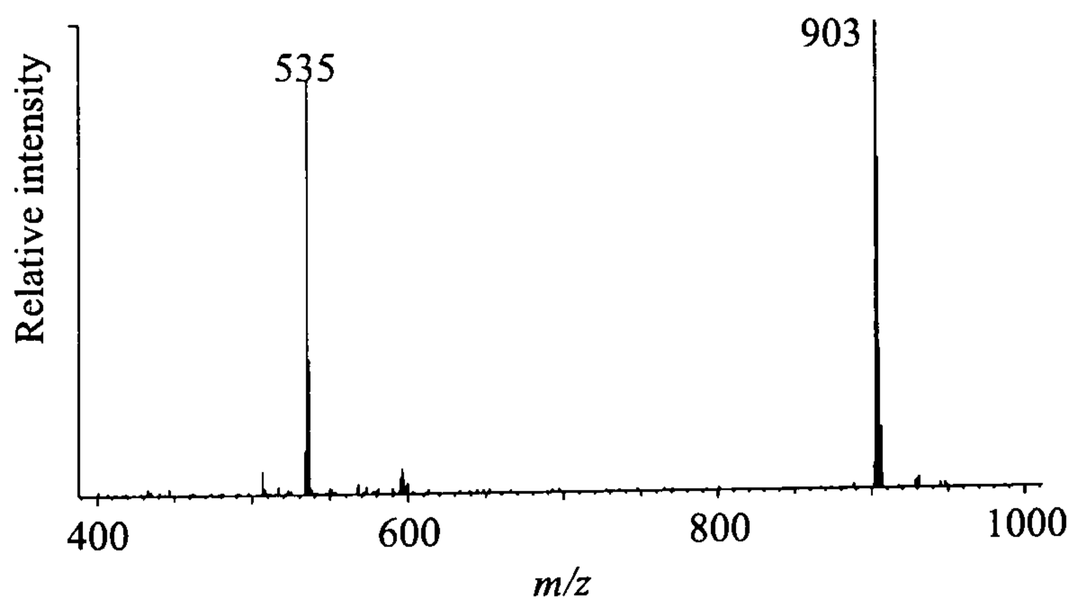


Figure 5-19. *Mass spectrum of SCE peak b (MH^+=903).*

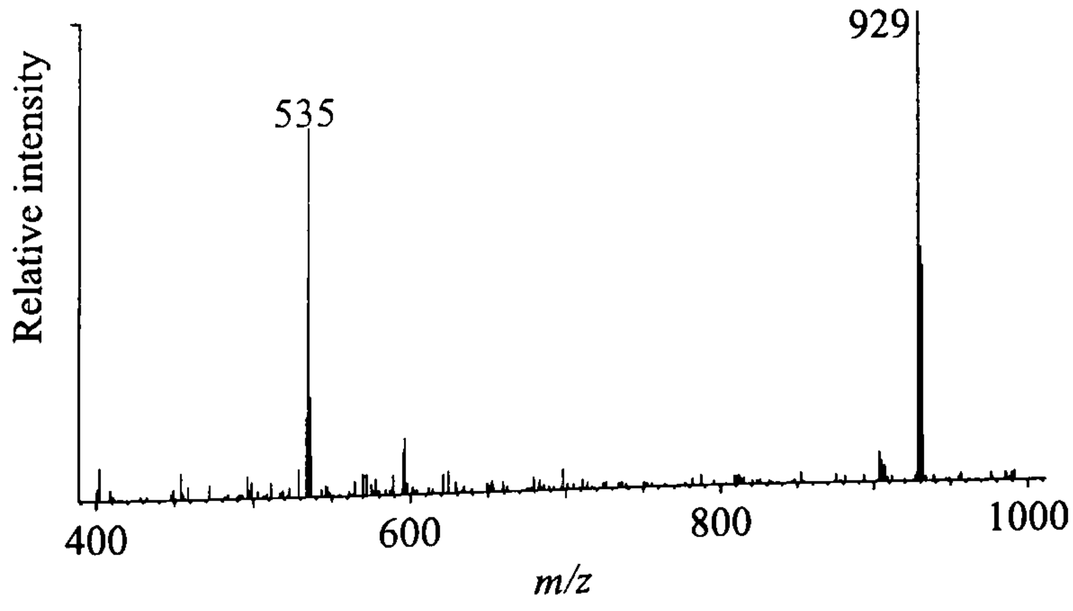


Figure 5-20. *Mass spectrum of SCE peak c (MH^+=929).*

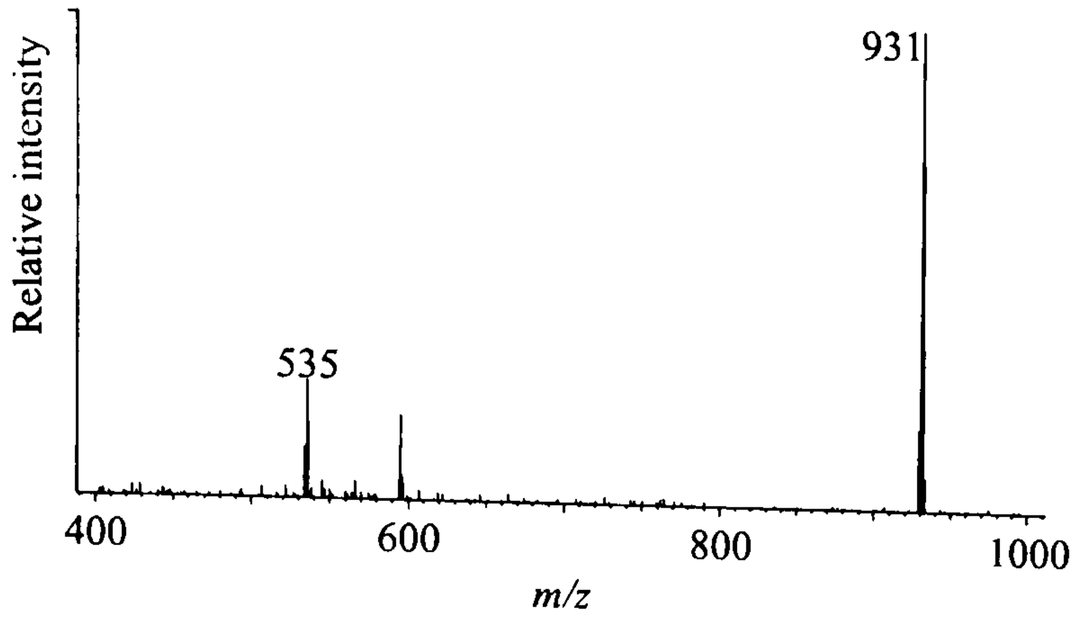


Figure 5-21. *Mass spectrum of SCE peak d (MH^+=931).*

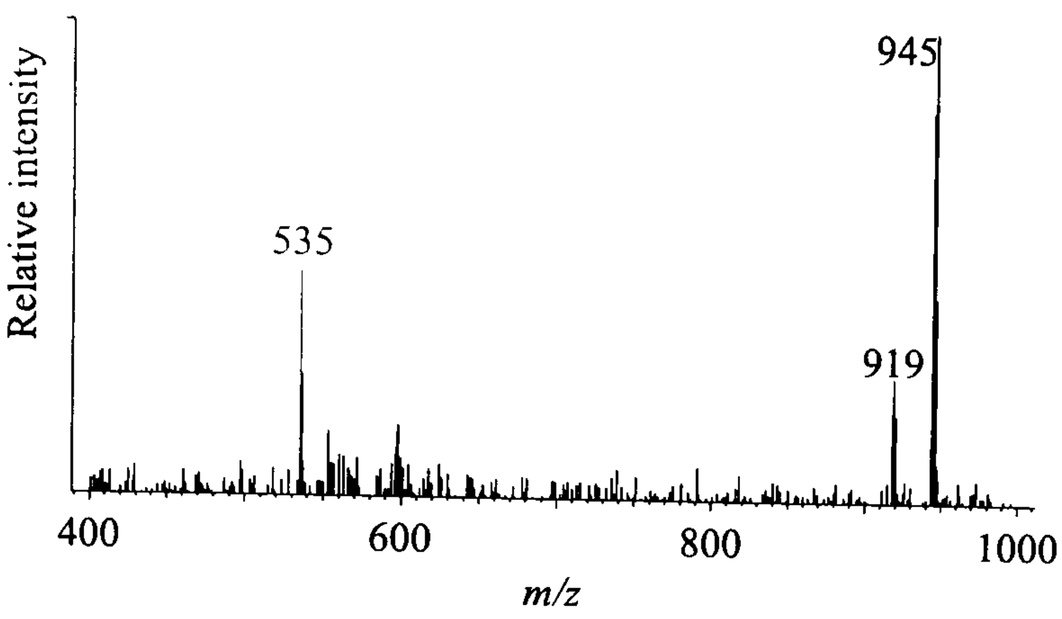


Figure 5-22. *Mass spectrum of SCE peak e (MH^+ = 919 and 945).*

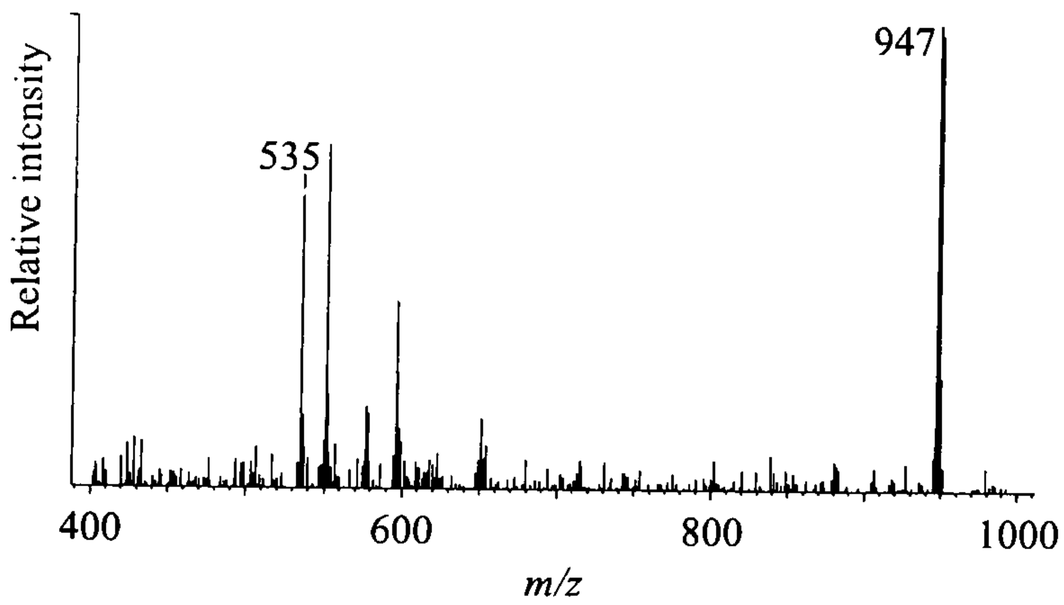


Figure 5-23. *Mass spectrum of SCE peak f (MH^+=947).*

5.4.8. Sterols

5.4.8.1. Starved Animals

The distribution (fig. 5-24) is dominated by cholest-5-en-3β-ol (peak 2, **A1**) along with cholesta-5,22-dien-3β-ol (1, **A3**) and cholesta-5,24-dien-3β-ol (4, **A2**) as expected; 5α-cholestan-3β-ol (3, **C1**) is also present in trace abundance. It should be noted, however, that there was no clear indication of the presence of the two C_{27} di-unsaturated sterols in the mass chromatograms (*m/z* 901; fig 5-17) as seen in the small scale experiment, so the SCEs are compared to the culture sterols only (see below).

5.4.8.2. Algal Culture

The distribution (figs. 5-24 and 5-25) contains a complex mixture of 10 sterols, three more than were observed in the small scale experiment (fig. 5-4). The 4-desmethyl sterols (see fig. 5-26 for mass spectra) are dominated by cholest-5-en-3β-ol (2, **A1**), a sterol commonly found in dinoflagellates and often the dominant component present (e.g. *Gonyaulax* spp.; Volkman, 1986). Other 4-desmethyl sterols present were 24-methylcholesta-5,22-dien-3β-ol (5, **A6**), 23,24-dimethylcholesta-5,22-dien-3β-ol (7, **A10**), 24-ethylcholesta-5,22-dien-3β-ol (8, **A9**) with 24-ethylcholest-5-en-3β-ol (10, **A8**) and 24-ethyl-5α-cholestan-3β-ol (11, **C8**) present in trace abundance. As expected, the 4-methyl sterols (see fig 5-27 for mass spectra) are dominated by 4α,23,24-trimethylcholest-22-en-3β-ol (dinosterol; 12, **B10**) and 4α,23,24-trimethylcholestan-3β-ol (dinostanol; 13, **B11**), with 4α,24-dimethycholest-22-en-3β-ol (9, **B6**) and 4α-methyl-5α-cholesta-3β-ol (6, **B5**) being minor components. Sterol assignments and structures are summarised in Table 5-4.

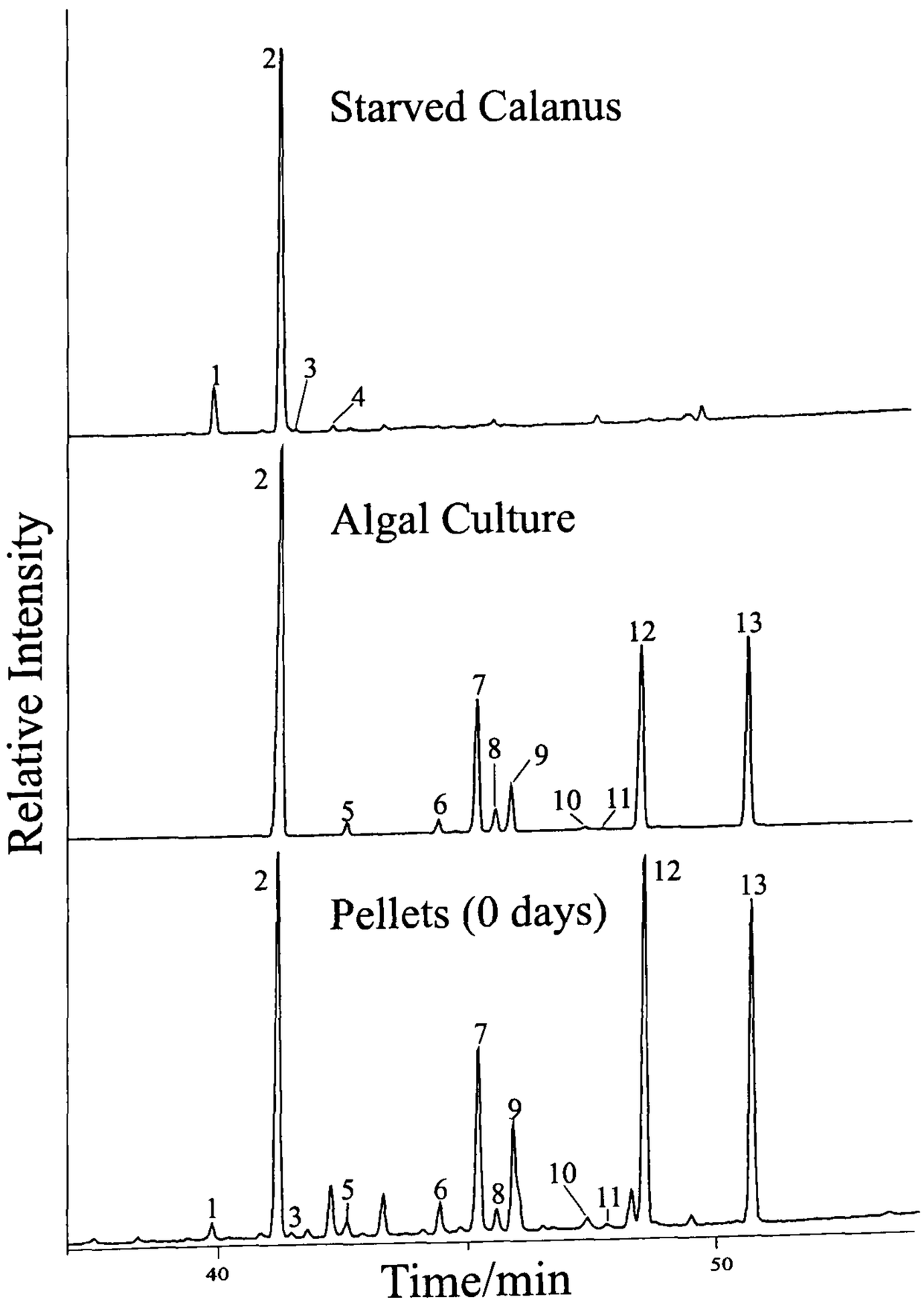


Figure 5-24. *Partial RIC traces of pre-starved animals, culture and faecal pellet free sterols (as TMSi ethers; unlabelled peaks are non-sterol).*

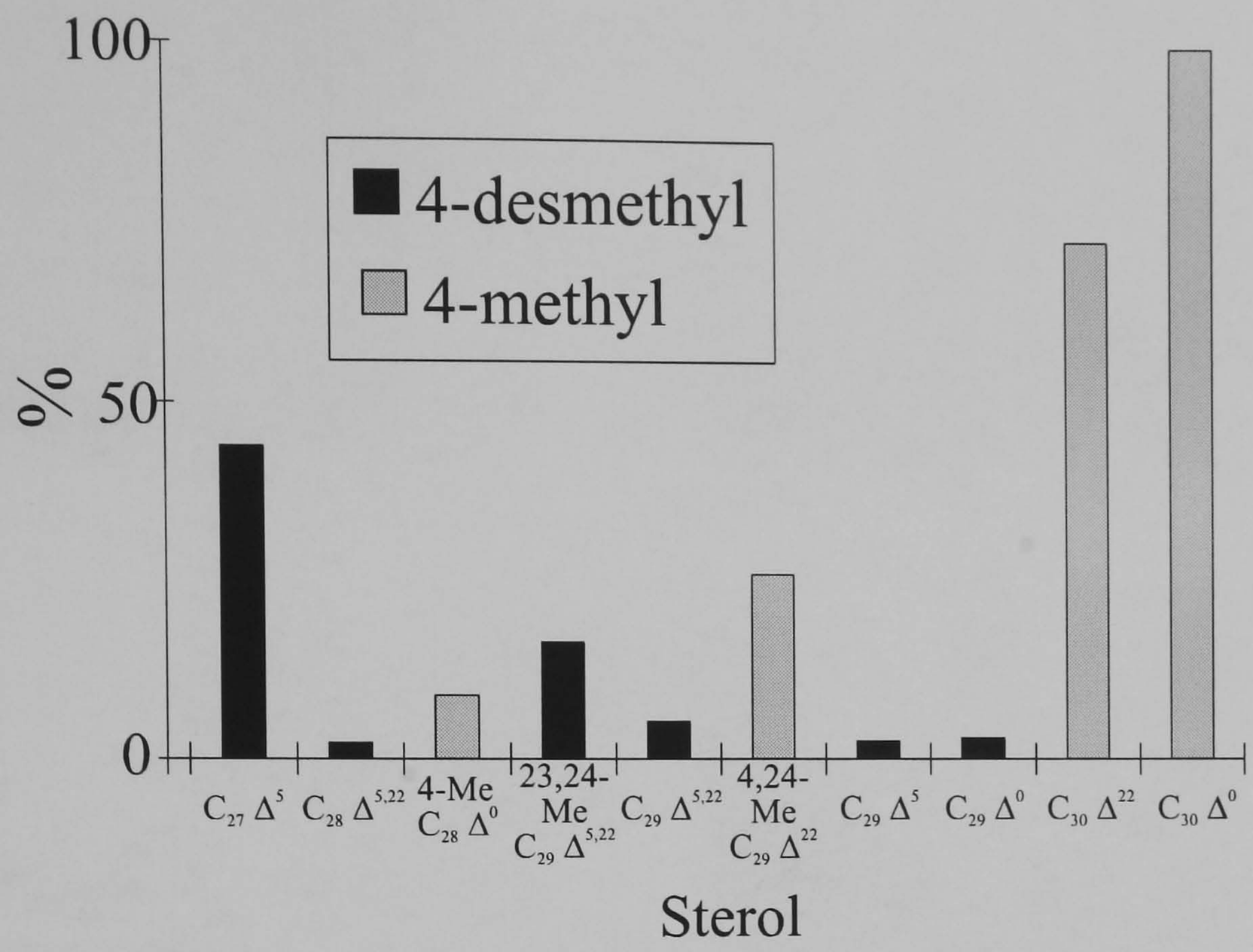


Figure 5-25. *P. micans culture free sterol distribution (* see fig. 5-24).*

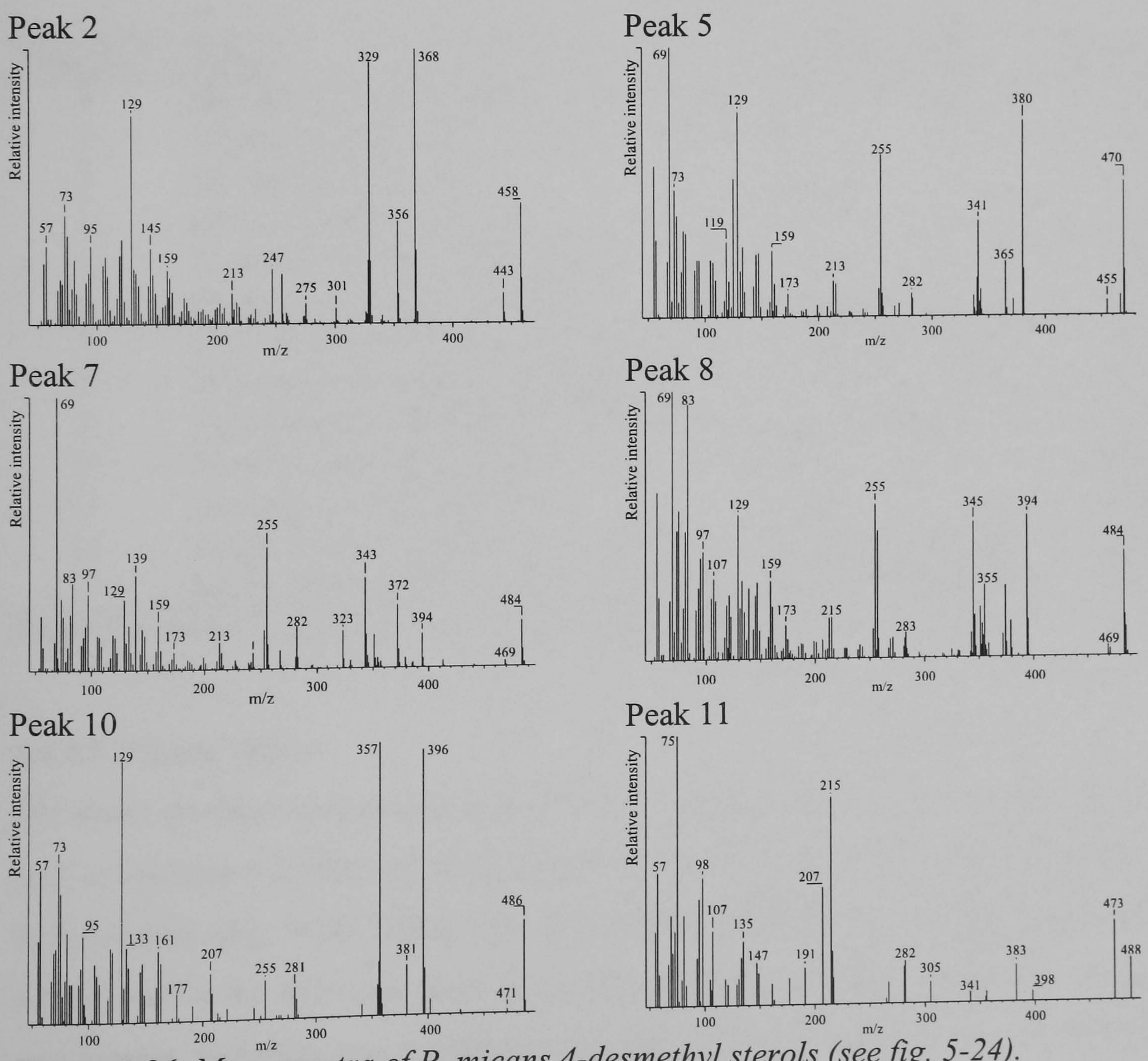
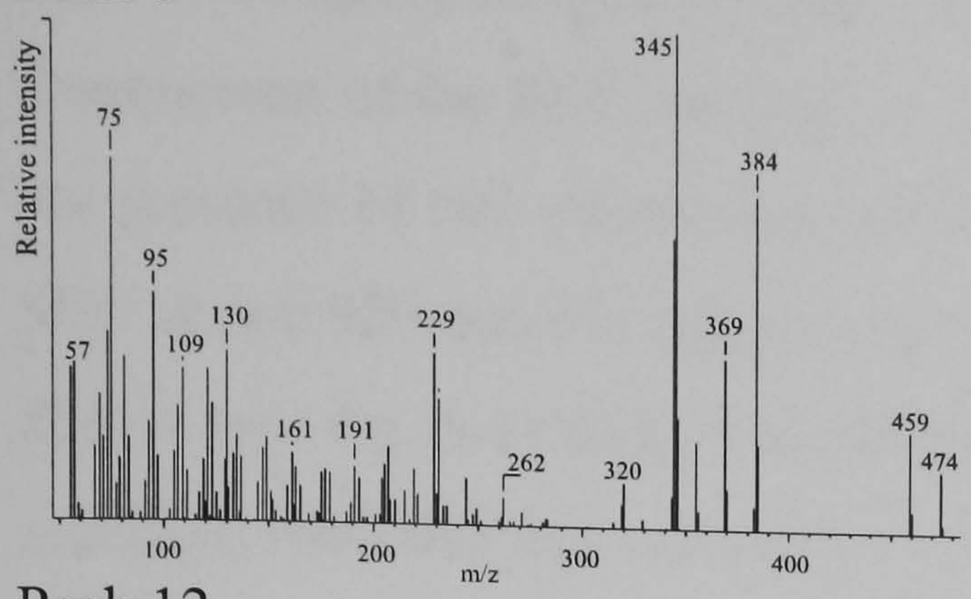
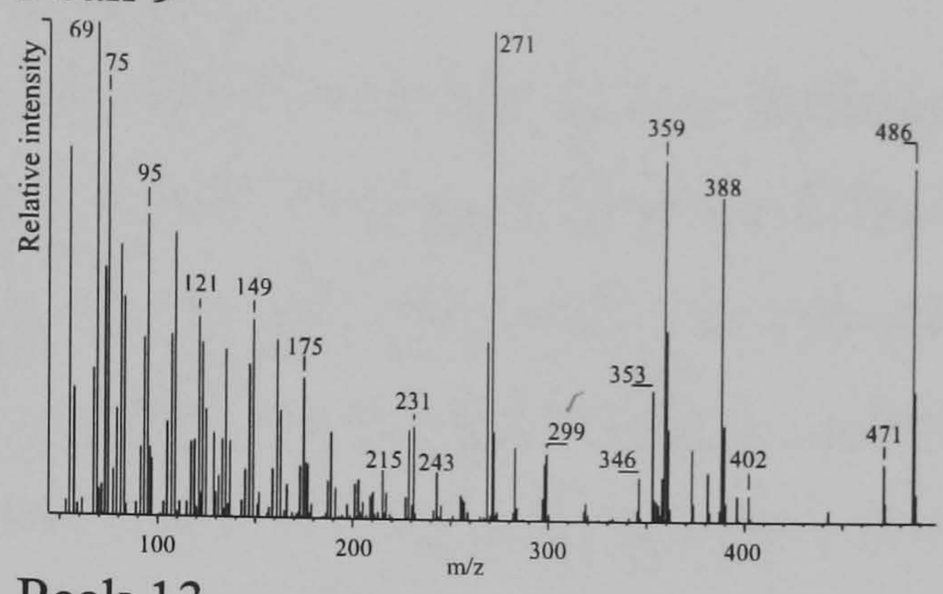


Figure 5-26. *Mass spectra of P. micans 4-desmethyl sterols (see fig. 5-24).*

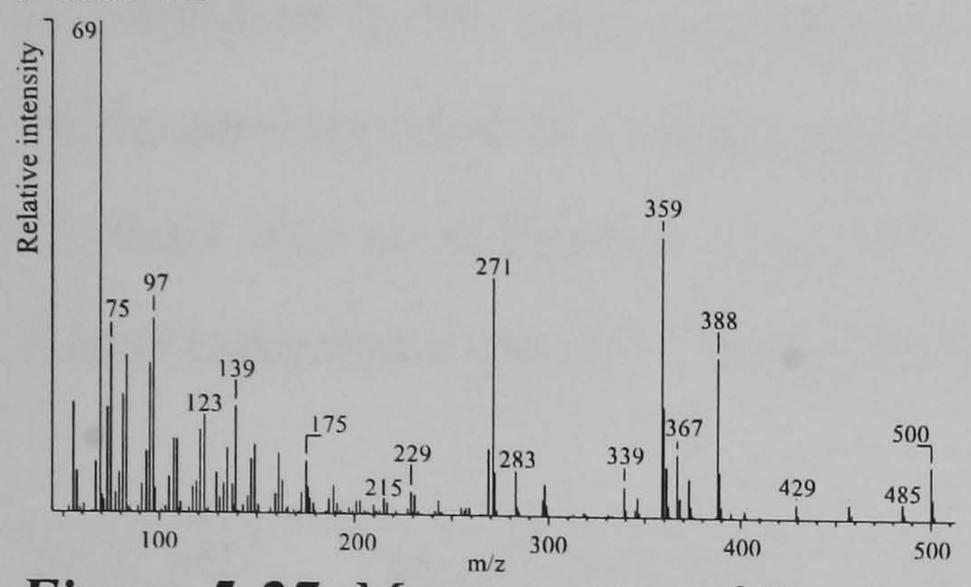
Peak 6



Peak 9



Peak 12



Peak 13

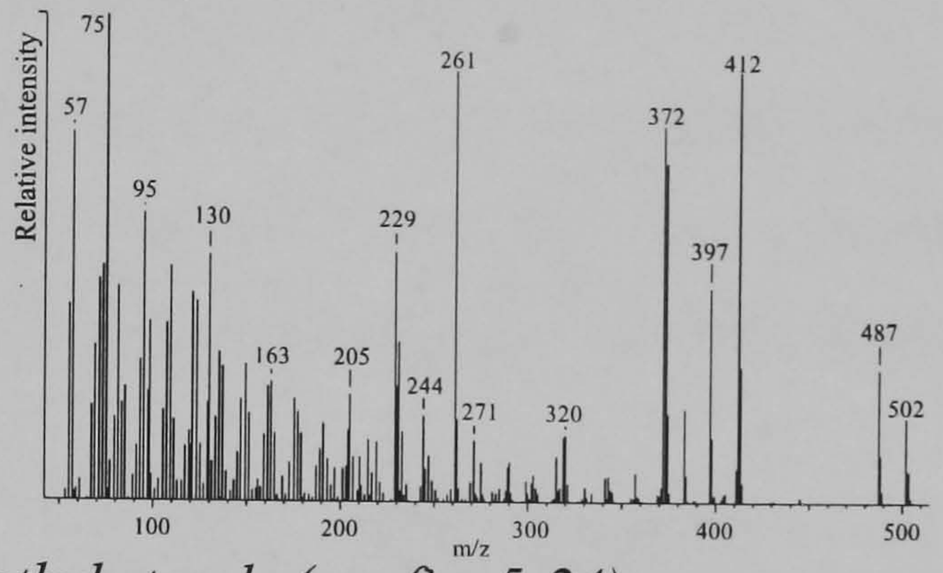


Figure 5-27. *Mass spectra of P. micans 4-methyl sterols (see fig. 5-24).*

Peak no.*	Sterol	Abbreviation	Structure
1	cholesta-5,22-dien-3β-ol	$C_{27}\ \Delta^{5,22}$	A3
2	cholest-5-en-3β-ol	$C_{27}\ \Delta^{5}$	A1
3	5α-cholestan-3β-ol	$C_{27}\ \Delta^{0}$	C1
4	cholesta-5,24-dien-3β-ol	$C_{27}\ \Delta^{5,24}$	A2
5	24-methylcholesta-5,22-dien-3β-ol	$C_{28}\ \Delta^{5,22}$	A6
6	4α-methyl-5α-cholestan-3β-ol	4-Me $C_{28}\ \Delta^{0}$	B1
7	23,24-dimethylcholesta-5,22-dien-3β-ol	23,24-Me $C_{29}\ \Delta^{5,22}$	A10
8	24-ethylcholest-5,22-dien-3β-ol	$C_{29}\ \Delta^{5,22}$	A9
9	4α,24-dimethylcholest-22-en-3β-ol	4,24-Me $C_{29}\ \Delta^{22}$	B6
10	24-ethylcholest-5-en-3β-ol	$C_{29}\ \Delta^{5}$	A8
11	24-ethyl-5α-cholestan-3β-ol	$C_{29}\ \Delta^{0}$	C8
12	4α,23,24-trimethylcholest-22-en-3β-ol	4,23,24-Me $C_{30}\ \Delta^{22}$	B10
13	4α,23,24-trimethyl-5α-cholestan-3β-ol	4,23,24-Me $C_{30}\ \Delta^{0}$	B11

Table 5-4. *Sterol assignments and structures (*see fig. 5-24).*

5.4.8.3. Faecal Pellets

The sterol distribution is similar to that of the culture, containing all 10 algal sterols, as well as cholesta-5,22-dien-3β-ol (**A3**) and 5α-cholestan-3β-ol (**C1**) which were present in the animal (fig. 5-24). There was no indication of cholest-5,24-dien-3β-ol (peak 4, **A2**) present in the animal in trace abundance. There were also a number of non-sterol components, including one co-eluting with peak 9 which could not be resolved, so the abundance of this sterol in the pellets is overestimated (see below).

5.4.8.4. Comparison of SCE and Culture Free Sterols

Comparison of the SCE and free C_{29} mono and diunsaturated sterols is complicated by the presence of two sterols each of molecular weight corresponding to the SCEs with MH^+ at *m/z* 929 and 931 (peaks c and d in fig. 5-17). As each sample was only a small aliquot (see fig. 5-1) there was insufficient material to isolate the sterols by hydrolysis or $LiAH_4$ reduction and analysis by GC and GC-MS, so in each case the SCE abundance is compared to the total available sterol of the appropriate mass (fig. 5-28). All other SCEs corresponded to a single available sterol and were assigned accordingly (Table 5-5); there was no indication of an SCE with MH^+ at *m/z* 933 corresponding to the minor stanol component (peak 11 in fig. 5-24), presumably as it was below detection limits.

SCE MH^+	Esterifying sterol	Sterol structure
903	cholest-5-en-3β-ol	A1
915	24-methylcholesta-5,22-dien-3β-ol	A6
919	4α-methyl-5α-cholestan-3β-ol	B1
929	23,24-dimethylcholesta-5,22-dien-3β-ol	A10
	24-ethylcholesta-5,22-dien-3β-ol	A9
931	4α,24-dimethylcholes-22-en-3β-ol	B6
	24-ethylcholest-5-en-3β-ol	A8
945	4α,23,24-trimethylcholest-22-en-3β-ol	B10
947	4α,23,24-trimethyl-5α(H)-cholestan-3β-ol	B11

Table 5-5. *SCE MH^+ and corresponding esterified sterol.*

Comparison of the SCE and culture sterol distributions again shows a significant decrease in the abundance of 4-methyl sterols in the SCEs relative to the culture (fig. 5-28).

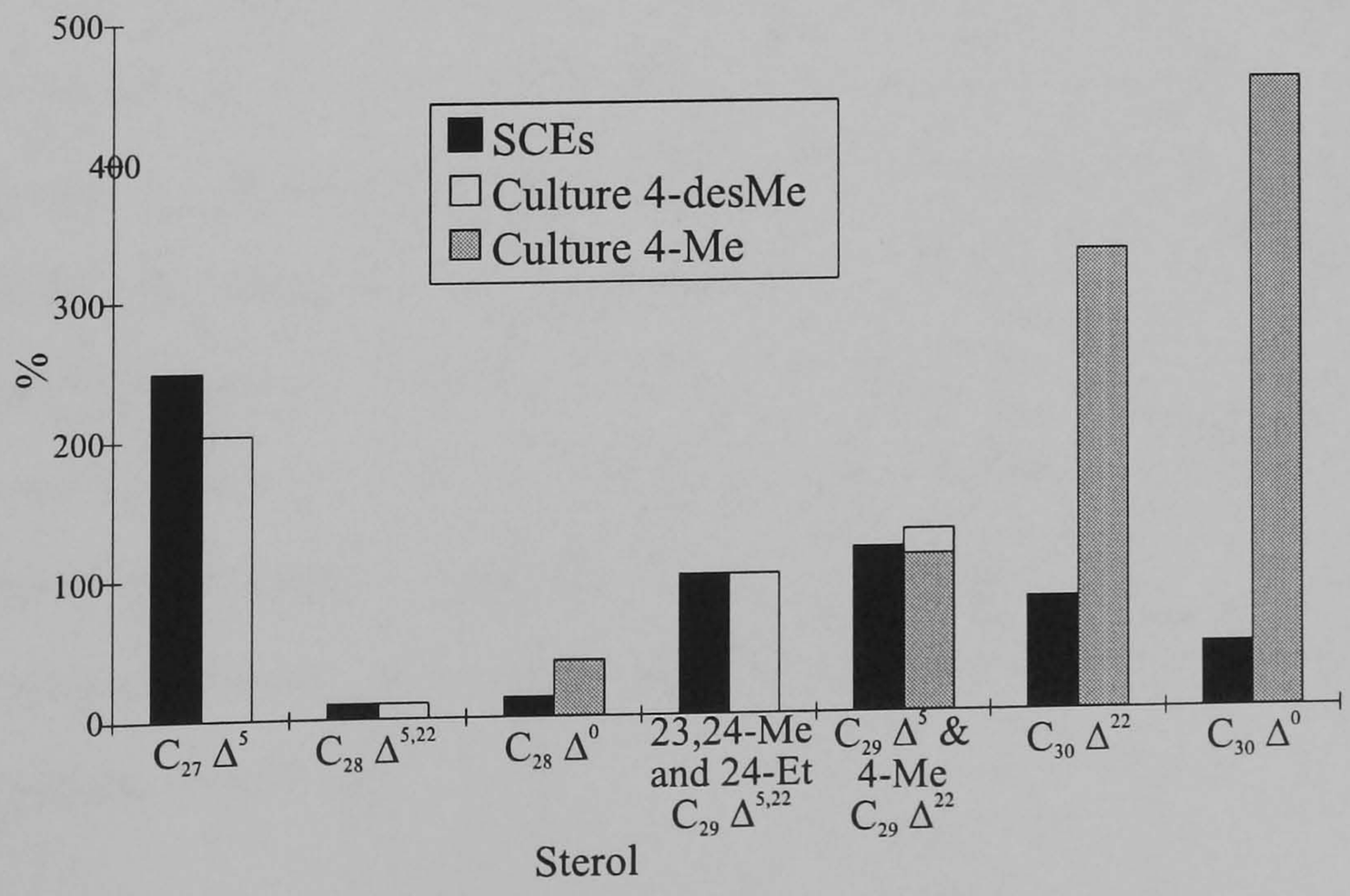


Figure 5-28. *Comparison of SCE and culture free sterol distributions.*

5.4.9. Ageing studies

5.4.9.1. SCEs

Comparison of the relative abundance of the SCEs at the three stages of ageing and in the sterilised pellets shows no significant change in the overall distribution over the 29 day period (fig. 5-29).

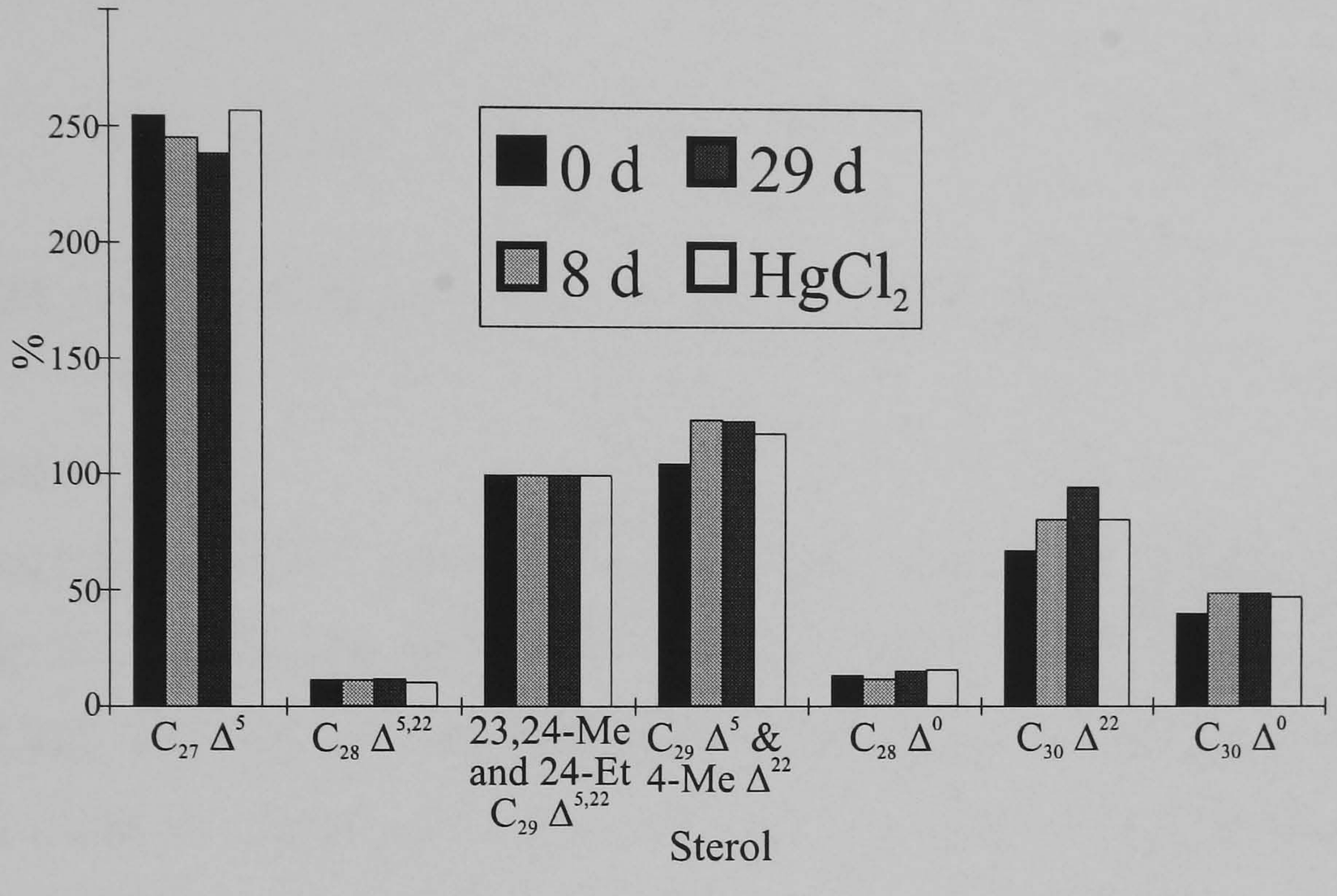


Figure 5-29. *Faecal pellet SCE relative abundance with ageing (based on peak areas in HPLC 400 nm chromatogram).*

The mass of total SCEs (as pyrophaeophytin *a* equivalents; fig. 5-30a) was calculated relative to the internal standard and showed an initial drop surprisingly followed by a rise for the 29 d sample. This is considered to be unlikely given the results for the similar study with a diatom (see Chapter 4). A *t*-test revealed no difference between the mean masses measured for the 8 and 29 d samples (95% confidence limits). This is thought to be due to inaccuracy in the method used to separate the pellet aliquots (see below and Chapter 8). However, the SCEs contribution to the total chlorins (fig. 5-30b) does agree with the results in Chapter 4 in that the SCEs are more stable relative to total chlorins, increasing from *ca.* 17% to 31% (8 d) and 59% (29 d). The SCE abundance in the sterilised pellets is slightly higher than that of the fresh pellet sample (*ca.* 30% *vs.* 17%) presumably due to the absence of pyrophaeophorbide *a* from the total chlorins in the sterilised pellets (fig. 5-16).

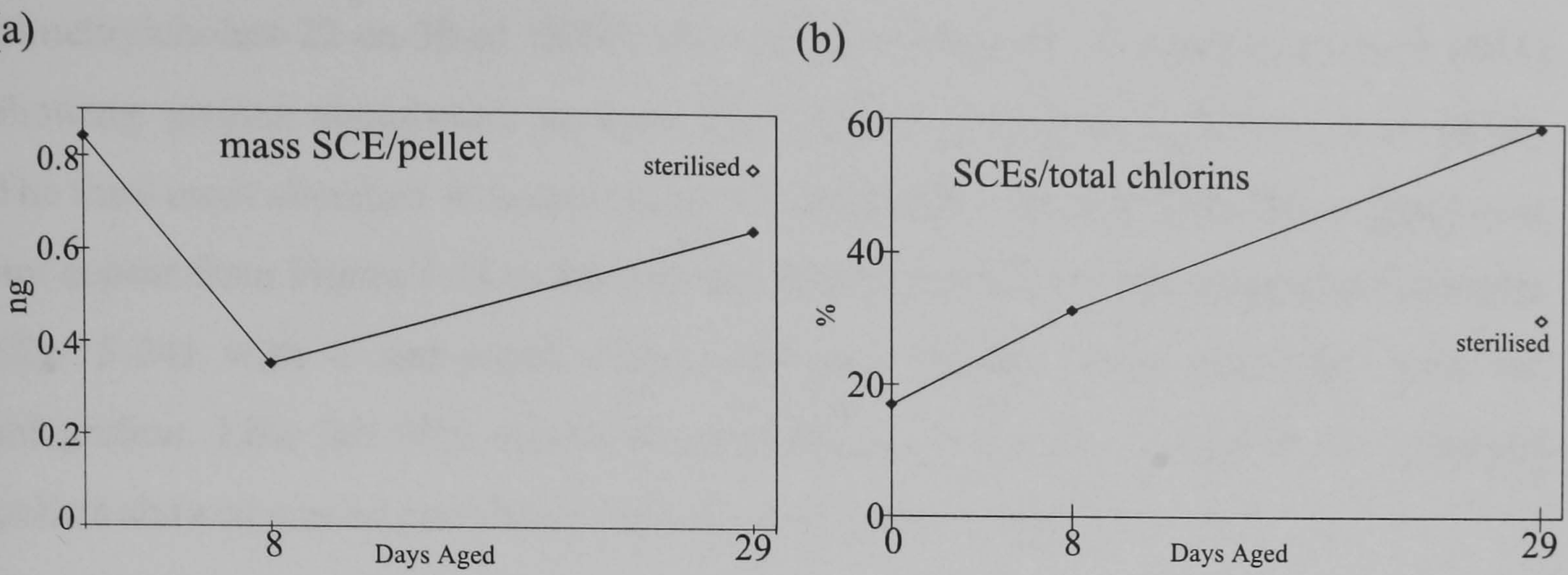


Figure 5-30. *(a) Mass SCEs per pellet; (b) SCEs % of total chlorins.*

5.4.9.2 Sterols

Comparison of the relative abundance of the pellet free sterols over the first 8 d of ageing (fig. 5-31) shows that there is little change in the major algal sterols (cholest-5-en-3β-ol [**A1**], 24-methylcholesta-5,22-dien-3β-ol [**A6**], 23,24-dimethylcholesta-5,22-dien-3β-ol [**A10**], 4α,24-dimethylcholest-22-en-3β-ol [**B6**], 4α,23,24-trimethylcholest-22-en-3β-ol [**B10**] and 4α,23,24-trimethyl-5α-cholestan-3β-ol [**B11**]).

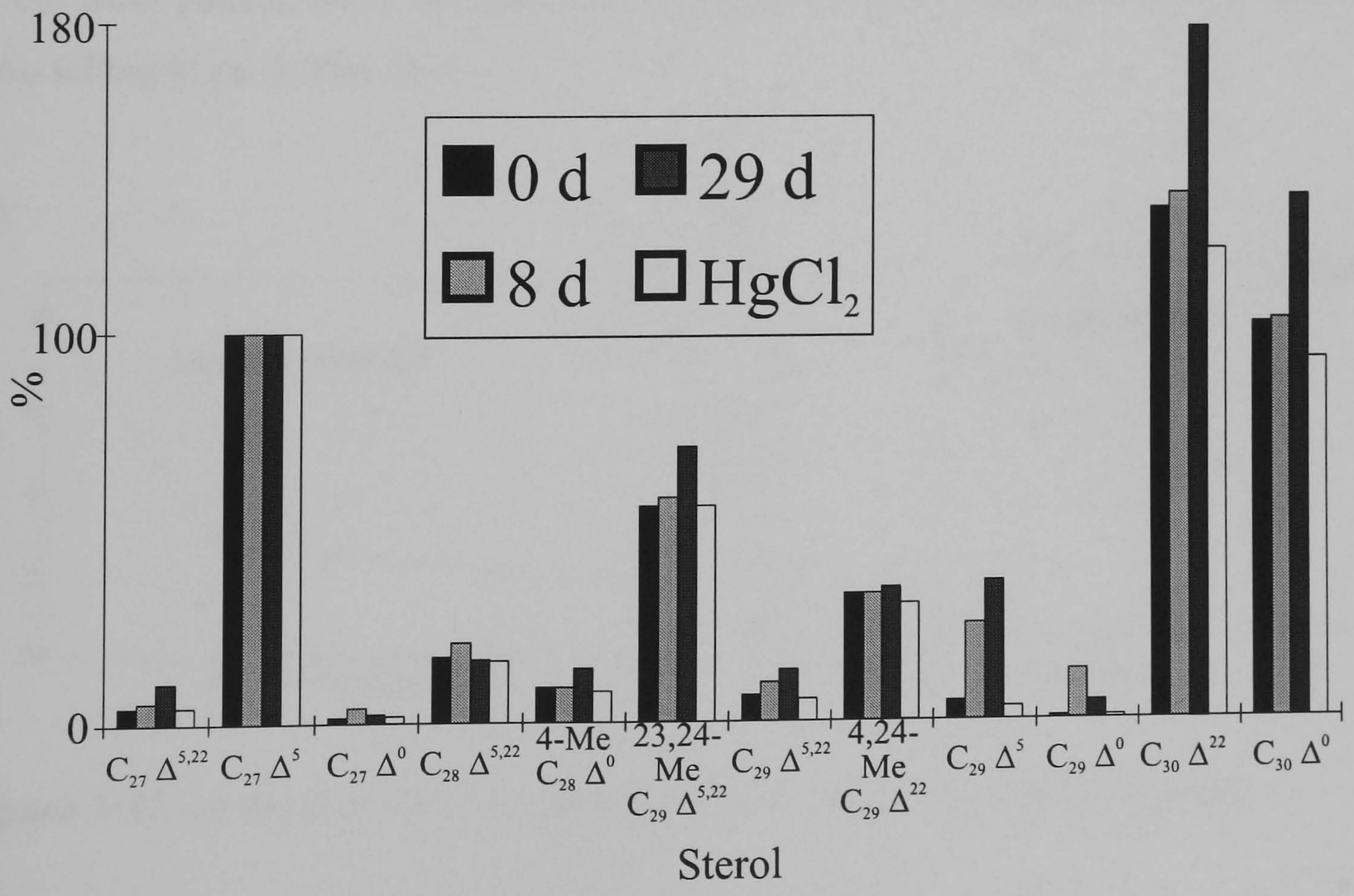


Figure 5-31. *Relative distribution of free sterols in faecal pellets with ageing.*

After 29 d there is some change with the two major 4-methyl sterols 4α,23,24-trimethylcholest-22-en-3β-ol (**B10**) and 4α,23,24-trimethyl-5α-cholestan-3β-ol (**B11**) showing greater abundance, as does 23,24-dimethyl-cholesta-5,22-dien-3β-ol (**A10**). The third most abundant 4-methyl sterol (4α,24-dimethylcholest-22-en-3β-ol [**B6**]) does not appear from Figure 5-31 to have shown the same effect but this component co-elutes (fig. 5-24) with a non-sterol component (see above) which interfered with the integration. Like the SCE sterols the distribution of the free sterols in the sterilised pellets showed a good correlation with those in the fresh pellets.

The total mass of free sterol per pellet showed the expected trend of continual reduction (*cf.* Harvey, 1987) in mass over the 29 d period (*ca.* 8 ng to 2 ng; fig. 5-32a); however, given the difficulty encountered with the SCE results (fig. 5-30a) it is likely that the actual reduction should be significantly greater. The mass of sterols remaining in the sterilised pellets was similar to that in the fresh pellet sample as might be expected.

Comparison of the mass of total sterols to total SCEs in the pellets (fig. 5-32b) indicates that, although the free sterols are significantly more abundant (*ca.* 1 order of magnitude) in the fresh pellets, SCEs are significantly more stable than the free sterols, with the ratio falling to *ca.* 3 after 29 d.

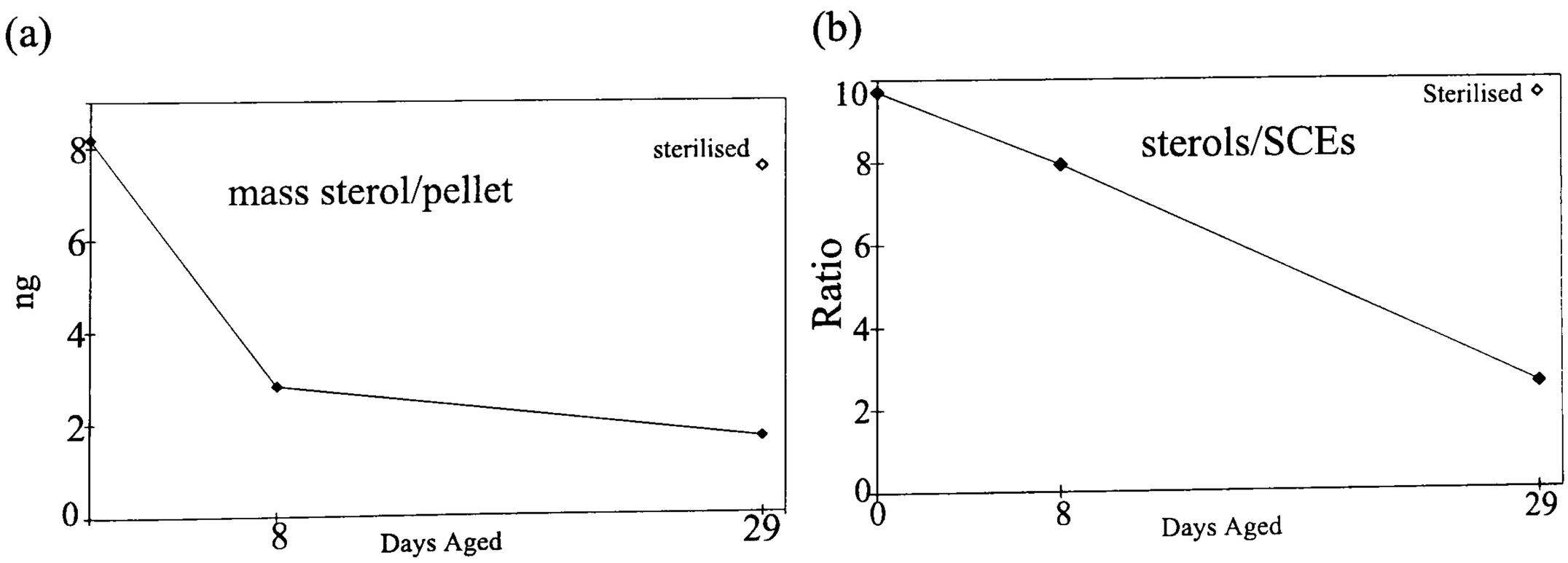


Figure 5-32. *(a) Mass of sterol per pellet, (b) Ratio of sterols to SCEs in pellets.*

5.5. DISCUSSION

5.5.1. Small Scale Experiments

The production of SCEs has been demonstrated during grazing on two members (*P. micans* and *A. tamarensis*) of a fourth major algal division (the Dinophyta). A preliminary, small scale experiment involving *C. helgolandicus* grazing on *P. micans* revealed the production of the common herbivory products phaeophytin *a* (**VIII**), pyrophaeophytin *a* (**XI**) and pyrophaeophorbide *a* (**X**). This is the first experiment during this work to show production of pyrophaeophorbide *a* in excess of pyrophaeophytin *a* (fig. 5-2), suggesting that the feeding algal cell concentration (700 cells ml^{-1}) was low and therefore led to more extensive chl transformation (*cf.* Downs, 1989 and Chapter 4).

A total of seven sterols were present in the alga, at least six of which were incorporated into the SCEs. The sterol distributions of the two fractions demonstrated clear discrimination against the incorporation of 4-methyl components into the SCEs (fig. 5-5). If the two C_{29} mono unsaturated sterols (4α,24-dimethy-5α(H)-cholestan-3β-ol and 24-ethylcholest-5-en-3β-ol) co-eluting as SCEs are excluded from the comparison, as their relative abundance in the SCEs is unknown, then the 4-methyl components represented *ca.* 44% of the available algal free sterols but only 26% of the SCE sterols. A similar effect was observed in the *A. tamarensis* experiment even though this organism contained only one 4-desmethyl sterol cholest-5-en-3β-ol (cholesterol) and one 4-methyl sterol (4α,23,24-trimenthyl-5α(H)-cholest-22-en-3β-ol, dinosterol, **B10**) with dinosterol representing 37% of the algal free sterols but only 22% of the SCE sterols which in this case were only trace components in the faecal pellet pigment distribution. This effect is discussed in more detail below.

5.5.2. Large Scale Experiment

The *P. micans* experiment was repeated on a significantly larger scale for investigation of changes in the distribution and abundance of both SCE and free sterols in the pellets

during ageing. Unlike the preliminary experiment, pyrophaeophorbide *a* (**X**) was not the most abundant chlorin (fig. 5-13) even though the experiment was carried out at the same food concentration, suggesting that in this case the extent of transformation could be due to differences in the nutritional status of the animals (i.e. previous long term feeding history; e.g. Penry and Frost, 1991) rather than the actual dietary concentration.

As in the small scale experiments, significant discrimination against the incorporation of 4-methyl sterols was apparent (fig. 5-28), with 4-methyl sterols comprising 72% of the available free sterols but constituting only 28% of the SCE sterols. It is suggested that this effect may be a result of a steric effect due to the presence of the 4-methyl group interfering with the enzyme efficiency and altering the rate of the esterification process, leading to a lower proportion of 4-methyl sterols in the SCE fraction relative to the source organism. Previous studies of a variety of sedimentary environments (King and Repeta, 1994; Pearce *et al.*, 1998) have found 4-methyl sterols to be significantly more abundant in the free fraction relative to the SCEs. This process ascribed to greater resistance of 4-methyl sterols to biodegradation, i.e. a greater lability of desmethyl sterols (King and Repeta, 1994; Pearce *et al.*, 1998; *cf.* Wakeham, 1987; Teece, 1994) and to microbial reduction of stenols to stanols (King and Repeta, 1994 and references therein). This implies in turn that esterification of sterols to the chlorin nucleus preserves the sterol distribution from selective changes which occur during biodegradation and consequently lead to overestimation of the input from dinoflagellates (and other 4-methyl sterol containing organisms) when free sedimentary sterols are used as indicators of phytoplankton community structure. It appears therefore, that the lower abundance of 4-methyl sterols within SCE fractions *vs*. free sterol fractions occurs in the natural environment as a result of two processes, the preferential biodegradation of free 4-desmethyl sterols and discrimination against the incorporation of 4-methyl sterols into SCEs during herbivory. Indeed in the present study, the former effect - preferential biodegradation of free desmethyl sterols - was seen clearly in the pellet ageing experiments, whereas the free sterol distribution did not change in the sterilised aged pellets.

Within the 4-methyl sterols in the large scale experiment the pellet free distribution and SCE distribution showed a significant difference compared with the culture free sterols. The abundance of the two major components changed such that dinostanol became less abundant than dinosterol in the pellets and their SCEs (fig 5-28). The same effect was seen to a lesser extent in the small scale *P. micans* experiment (fig. 5-5). Previous workers (Harvey *et al.*, 1989) have shown that dinosterol displays quantitative passage through copepod guts and is not retained in the animal as a result of assimilation. As ring saturated sterols, including dinosterol and dinostanol, have been shown to be more resistant to crustacean and microbial enzymatic processes in the copepod gut (Harvey *et al.*, 1989) it would be expected that both dinosterol and dinostanol should be present in faecal pellets in an abundance similar to that in the source organism, so the difference observed here in both the pellet free sterols and SCEs is difficult to explain, although the effect appears to be real, being seen in both experiments. There have been reports in which 4-methyl sterols have been removed during grazing as both 4-methyl and 4-desmethyl sterols containing a $\Delta^{8(14)}$ bond are preferentially removed as is 4α,22,23-trimethyl-cholest-17(20)-en-3β-ol (**B12**) (Harvey *et al.*, 1987), however some other effect is occurring here to produce the reduction in dinostanol.

Unlike the previous experiments in which cholesterol was absent or present as only a minor component in the diet (Chapters 3 and 4), there were no changes in the abundance of the other 4-desmethyl sterols, indicating that where present in an alga in sufficient quantity in the natural environment to provide the animal with its nutritional requirement of cholesterol the distribution of other 4-desmethyl sterols would not undergo alteration prior to esterification to pyrophaeophorbide *a* and hence in this type of situation the 4-desmethyl sterols would be better indicators of the algal substrate than in the situation where the distribution had been altered by assimilation by zooplankton.

Two of the three animal sterols (cholesta-5,22-dien-3β-ol [**A3**] and 5α(H)-cholestan-3β-ol [**C1**]) which were not present in the alga, were present in the pellets; however, there was no evidence of cholesta-5,24-dien-3β-ol (**A2**). This is in agreement with the observations of Harvey *et al.* (1987) who did not find this component in faecal pellets produced by *C. helgolandicus* fed the dinoflagellate *Scrippsiella trochoidea* at three

different food concentrations, presumably as it is retained for conversion to cholest-5-en-3β-ol (Goad, 1978, 1981). All of the algal sterols ingested (total=24; 15 4-methyl) in the Harvey *et al.* (1987) study were present in the pellets, with dinostanol showing a slight increase in relative abundances in pellets compared to the alga, the reverse of which was observed here. Cholesterol was also present in greater abundance, despite its occurrence as only a minor component in the *S. trochoidea* substrate.

Due to problems with the method used to separate the pellets into aliquots (dilution; *cf.* manual counting, Chapter 4) it was not possible to estimate accurately the change in total mass of SCEs in the pellets as they aged; however, the marked drop in concentration after 8 days taken with the results of the pellet ageing experiments in Chapter 4 does show that significant degradation occurs over a 30 d period. The similarity in SCE concentration in the fresh pellets and sterilised (29 d ageing) indicates that the degradation effect is a result of biodegradation by the bacterial community in the pellets. Nevertheless the sterol distribution within the SCEs remained constant, indicating that esterification to pyrophaeophorbide *a* protects both 4-methyl and 4-desmethyl sterols from biodegradation with equal efficiency. This is discussed in more detail in Chapter 7. As observed in Chapter 4, however, the abundance of the SCEs relative to total chlorins increased significantly from *ca.* 17% to 59% (fig. 30b) over the 29 d period (*cf. ca.* 8% to 16% in 30 d, large scale *T. weiss* experiment, Chapter 4).

As for the SCEs, it was not possible to determine accurately the total mass of free sterols in the aged pellets; however, the ratio of total sterols to total SCEs could be compared within each sample. There was a rapid decrease in the ratio between the two sterol fractions (fig. 5-32b) with the free sterols present initially an order of magnitude greater than the total mass of SCEs; however, after the 29 d period this had fallen to only 3 times more abundant than the SCEs. This indicates that the SCE sterol distribution is not only more stable than that of the free sterols but that SCEs themselves are significantly more stable than free sterols (see also above).

The pellet free sterol distribution underwent little alteration during the initial 8 d ageing period (fig. 5-31); after 29 d, however, there was an indication of reduction in

abundance of the major sterols relative to the two most abundant 4-methyl sterols. This provides further evidence against the use of free sedimentary 4-methyl sterols in estimating relative inputs from dinoflagellates.

The pigment signatures in the *A. tamarensis* experiments revealed the presence of some unexpected components in the culture, control and pellets. The absence of chl *a* and presence of pyrophaeophytin *a* (**XI**) in the culture suggests that the culture had reached stationary phase and had started to undergo senescence prior to feeding. This component has been observed previously in senescent systems (e.g. Louda *et al.*, 1998). The algal control, as well as containing pyrophaeophytin *a*, also contained both C-13^2 epimers of hydroxychlorophyllone (chlorophyllone; e.g. Sakata *et al.*, 1990; Harris *et al.*, 1995a). The presence of these components in a dinoflagellate culture represents the first observation of these compounds associated with a dinoflagellate. This bicyclic chlorin has previously only been associated with diatoms (e.g. Watanabe *et al.*, 1983), with the only exception to date being its presence as a minor component in the haptophyte *I. galbana* which was incorrectly assigned as a diatom (Sakata *et al.*, 1994). As well as chlorophyllone there was also evidence of 13^2,17^3-cyclophaeophorbide *a* enol (**XXXXIV**) originally detected in a sponge (Karuso *et al.*, 1986). This component has not been reported previously in marine algae although it has been found in sediment trap material (Peru Upwelling area) as well as being one of the major chlorin components in the Black Sea (Ocampo *et al.*, 1999a). It is considered to be the precursor of other sedimentary chlorins and porphyrins containing the seven+five exocyclic ring system such as chlorophyllone so it is not surprising to see both of these components together in the algal control. It was, however, absent from the faecal pellets, whereas chlorophyllone was again present as a minor component. Another novel component, 13^2-oxopyrophaeophytin *a* (**XXXXIIIb**; see Chapter 4) was also observed in both the control and the pellets, and in the pellets from the large scale *P. micans* experiment. This supports the previous suggestion that both it and the epimers of chlorophyllone were only detected in faecal pellets (large scale *T. weiss* experiment, Chapter 4) due to an enhanced stability relative to other chlorins such as phaeophytin *a* and phaeophorbide *a*.

5.6. Summary

The production of SCEs during grazing on two member of a fourth major algal division (the Dinophyta) has been confirmed with *C. helgolandicus* grazing on *P. micans* and *A. tamarensis*. Both 4-desmethyl and 4-methyl sterols were incorporated into the SCEs. There was, however, significant discrimination against the uptake of 4-methyl components in both cases, which is thought to be a result of steric hindrance due to the presence of the 4-methyl group. This is the first time that this effect has been observed directly in the laboratory and provides a second mechanism by which the relative abundances of sedimentary 4-methyl SCE sterols are lower than free 4-methyl sterols which are known to be more resistant to degradation than free desmethyl sterols. This means that, whereas using free 4-methyl sterols as indicators of phytoplankton community structure can overestimate the contribution from dinoflagellates, use of SCE 4-methyl sterols would underestimate their contribution. On the other hand, however, given that once esterified, SCE sterol distributions are more stable than free sterol distributions, so use of SCE sterols would provide a more accurate indicator of phytoplankton community then the corresponding free sedimentary sterols. This is discussed further in Chapter 7.

A number of chl transformation products, previously only associated with diatoms, have been observed in a senescent algal culture (*A. tamarensis*, dinoflagellate) and in faecal pellets derived therefrom, one of which was also present in pellets derived from the dinoflagellate *P. micans*. These findings represent a new source for such components which have been found to be amongst the most abundant chlorin components in certain sedimentary environments and indicates that pigments of this type are not unique indicators of diatoms.

Chapter 6

FEEDING EXPERIMENTS WITH A HETEROTROPHIC DINOFLAGELLATE AND A SMALL MESOZOOPLANKTON POPULATION

6.1. INTRODUCTION: MICROZOOPLANKTON EXPERIMENTS

6.1.1. Background

Most studies of the transformation and degradation of chl in the aquatic environment which result from herbivorous grazing have concentrated almost exclusively on macro-zooplanktonic herbivores, particularly copepods (e.g. Shuman and Lorenzen, 1975; Hallegraeff, 1981; Head and Harris, 1992) and salps (e.g. Hallegraeff, 1981; Nelson, 1989). Studies of chl transformation by protozoa (microzooplankton) are far less common (see below), but it is now recognised that they are abundant components of both oligotrophic and eutrophic waters and play an important role in aquatic food webs (e.g. Barlow *et al.*, 1988). Heterotrophic flagellates, for example, have been found to play important roles in grazing on bacteria and small phytoplankton and in removing nutrients within the microbial food web (Goldman *et al.*, 1989), often being responsible for removal of high proportions of the phytoplankton standing stock. Heinbrokel and Beers (1979) demonstrated that up to 40% of total primary production can be grazed away by ciliates (tintinnids) in the southern California Bight and up to 65% is removed by microzooplankton grazing in the Celtic Sea in autumn (Burkill *et al.*, 1987).

The mechanisms which control the phytoplankton biomass in the nutrient rich areas of the open oceans are not well understood. However, heterotrophic dinoflagellates have been shown to be significant components of the microzooplankton in marine waters, being abundant in the North Atlantic and Subarctic North Pacific as well as in nearshore waters (Strom, 1991). High levels of pigment transformation products in suspended particulate matter observed in many parts of the ocean suggest a source other than rapidly sinking crustacean faecal pellets and could be due in part to microzooplankton grazing as the detrital particles they produce are not generally considered to have high enough sinking rates to be transported out of the photic zone (e.g. Strom 1993 and references therein). However, there have been reports of so called "minipellets" (<50 μm) which comprise a large proportion of total pellet flux in the eastern tropical Pacific and are thought to be produced by radiolaria (Gowing and Silver, 1985). Also Nöthig and von Bodungen (1989) reported that a large proportion of the Antarctic faecal pellet

flux is contributed by protists and more recently Buck and Newton (1995) have reported that pellets produced by the heterotrophic dinoflagellate *Gyrodinium* sp. account for *ca.* 29% of the total pellet flux at 50m in Dabob Bay, Washington.

Reports concerning the extent and type of chl transformation resulting from protozoan grazing vary widely and often are conflicting. In an early study phaeophytin *a* and phaeophorbide *a* appeared after grazing by a phagotrophic flagellate (Daley, 1973). An increase in chlorin products was also observed in a similar study by Goldman and Caron (1985) and SooHoo and Kiefer (1982) suggested that production of chlorins during grazing was the dominant pathway for chl loss occurring in microzooplankton, reporting values of 50-81% molar conversion of chl *a* to chlorins by natural marine microzooplankton samples, an assumption also made by Welschmeyer and Lorenzen (1985). All of the above results suggest that the impact of grazing by microzooplanktonic protozoa can be determined by monitoring changes in chlorin concentration. However, other studies found no change in the concentrations of chlorins (Landry and Hassett, 1982) and in experiments in which oligotrich ciliates were the dominant zooplankton species no increase in the concentrations of phaeophytins or phaeophorbides was observed, suggesting that, contrary to the above reports, the pathway of chl degradation in microzooplankton is either different from, or more complete than that found in macrozooplankton (Barlow *et al.*, 1988).

The widespread marine heterotrophic dinoflagellate *Oxyrrhis marina*, which is found in high abundance in rock pools, salt lakes, the Western Baltic, the English Channel and the Mediterranean Sea (Hansen *et al.*, 1996), is commonly used in protozoan grazing experiments. In an early study with *O. marina* and a cryptophyte (*Rhodomonas* sp.) no indication that chl *a* was converted to chlorins (phaeophytins and phaeophorbides) was observed and it was proposed therefore that chl *a* was converted directly to colourless residues, suggesting in turn that the activity of enzymes responsible for the production of colourless products was higher than those which produce chlorins (Klein *et al.*, 1986). Many protozoa including *O. marina* are phagotrophic, engulfing whole cells into food vacuoles which are initially acidic (pH normally 3-4.5; Klein *et al.*, 1986), but after a relatively short period following ingestion the pH rises to neutral then the pH becomes

alkaline, producing a significantly different environment to that encountered in the guts of crustaceans (*ca.* pH 6.8, *C. helgolandicus*; Pond *et al.* 1995); hence cells ingested by protozoa experience a more rapid change and wider range of conditions than cells ingested by copepods, which could perhaps explain differences in the degradation pathway. It has subsequently been suggested that the predator:prey ratios used in the Klein *et al.* (1986) experiments were not representative of the natural environment, so the almost total chl *a* removal could not be considered representative (Barlow *et al.*, 1988).

In a series of feeding experiments involving 5 species of ciliate and one heterotrophic dinoflagellate feeding on a variety of phytoplankton diets including the haptophytes *I. galbana* and *E. huxleyi*, green algae and dinoflagellates, transformation products of chls *a, b* and *c* were observed during every experiment, with the conversion of initial chl *a* to chlorins ranging from 7 to 93%, the highest values being observed near the beginning of an experiment with ratios (chl *a*:chlorins) then typically falling over time, possibly due to changes in digestion efficiency and ingestion of faecal material (Strom, 1993). As protozoa have intracellular digestion they do not produce discrete faecal pellets like those of copepods, but several studies have shown that some detrital material (e.g. minipellets; see above) can accumulate during protozoan herbivory (e.g. Gowing and Silver, 1985; Nöthig and von Bodungen, 1989; Buck and Newton, 1995). Production of chlorins by the heterotrophic dinoflagellate was consistently lower than with all of the ciliates in the experiments of Strom (1993). It was also observed that, in contrast to results for copepod feeding experiments, the ratio of phaeophytin *a*:phaeophorbide *a* tended to be high, suggesting that digestion of chl *a* is less thorough in protozoa than in copepods (but see below).

6.1.2. Present Study

Although the situation is unclear, certain herbivorous protozoa are clearly capable of producing chlorophyll biotransformation products, suggesting *a priori* that they may also be capable of producing SCEs. In order to investigate the potential for SCE production by protozoa, the heterotrophic dinoflagellate *O. marina* was cultured along

with an uncharacterised green alga which was present in the stock culture. This organism was chosen as it is one of the few readily available herbivorous protozoa that is easy to work with (Harris, personal communication). The heterotroph grazed on the chlorophyte at a rate comparable with the growth rate of the chlorophyte, so the culture was maintained in an active state simply by addition of further aliquots of culture medium. When the culture had been established and growing for *ca*. 4 weeks, samples of the total mixture were collected by filtration on to GF/F filters. Several studies have shown that *O. marina* does not excrete pigments into the culture medium (Klein *et al*., 1996, Barlow *et al*., 1988), so no samples of the filtrate were extracted. As a monoculture of the green algal diet was not available since it was an integral part of the heterotrophic dinoflagellate culture, separation of aliquots of the total mixture was achieved by filtration, producing one fraction containing the green alga only and a second fraction containing the heterotrophic cells plus a small proportion of the green alga. An outline of the experiment is given in Figure 6.1.

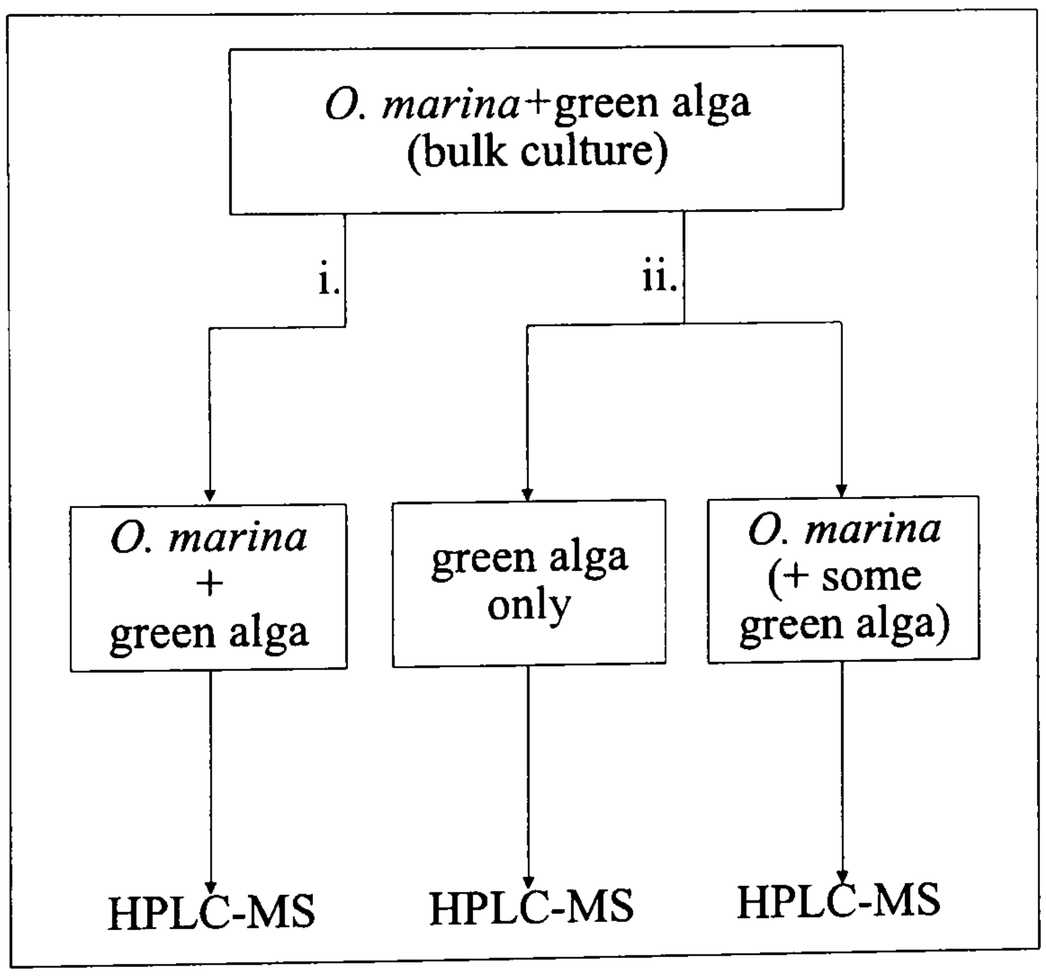


Figure 6-1. *Outline of O. marina and green alga experiment. (Key: i. total mixture filtered directly; ii. aliquot separated into 2 fractions).*

A second experiment was set up in which dinoflagellate cells were separated from the above bulk culture (*O. marina* and green alga) and transferred to a culture of *Isochrysis galbana*. As *O. marina* was found to graze on *I. galbana* at a significantly greater rate than that with the green alga, the mixture was maintained by addition of aliquots of fresh algal culture *ca*. every 3 days until a bulk culture of *ca*. 4 L was established. The total culture mixture was fed and then divided into 2 equal volumes 8 d prior to

harvesting. No fresh culture was added to one aliquot but was added to the second 2 d prior to harvesting.

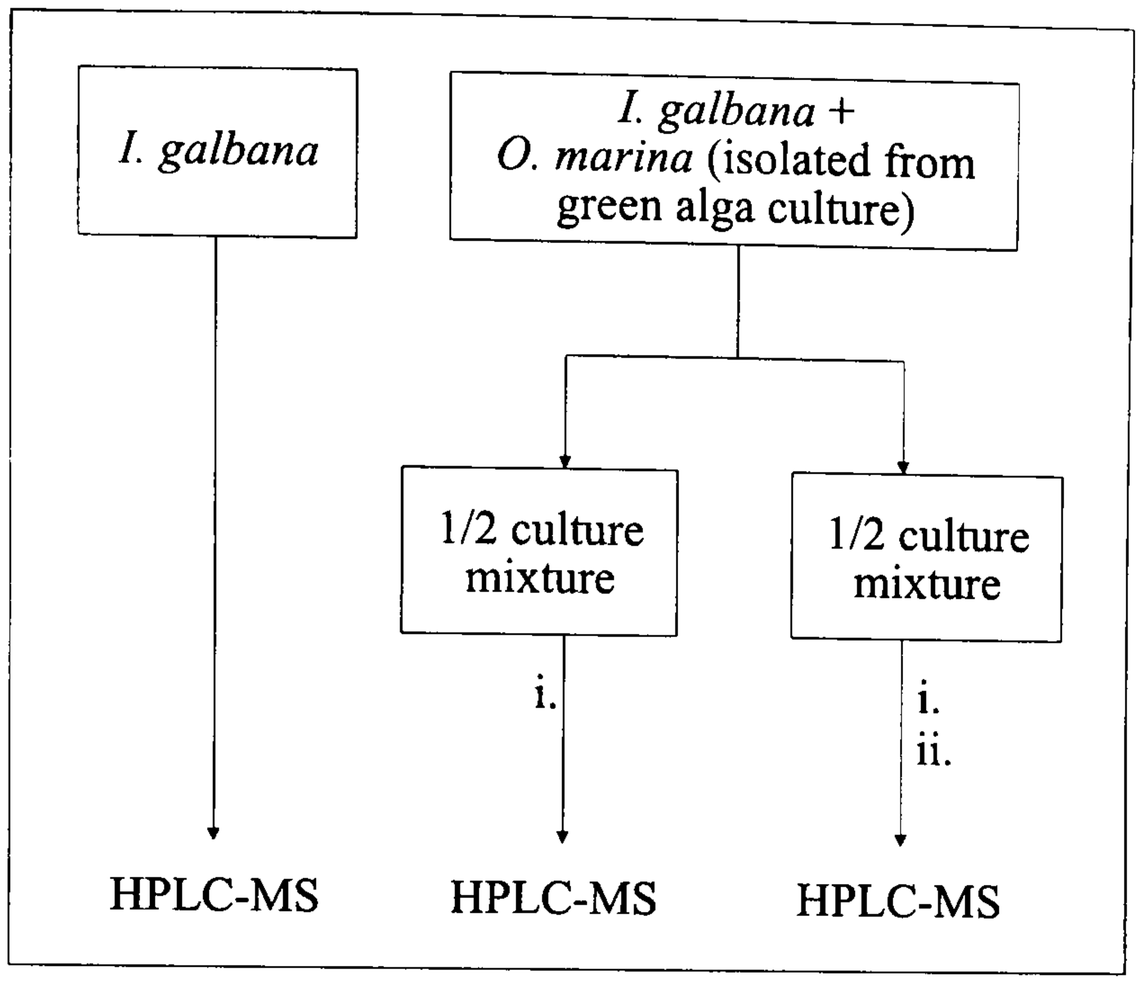


Figure 6-2. *Outline of O. marina and I. galbana experiment. (Key: i. Fed 8 d prior to harvesting; ii. fed again 2 d prior to harvesting.)*

6.2. INTRODUCTION: MESOZOOPLANKTON EXPERIMENT

6.2.1. Background

Harradine *et al.* (1996a) reported the presence of two triterpenoid chlorin esters as well as SCEs in a highly eutropic lake (Lake Valencia, Venezuela). As in the majority of "normal" SCEs the chlorin moiety was pyrophaeophorbide *a* (**X**). The first component was found to contain tetrahymanol (**XXIII**) which is synthesised by some classes of ciliated protozoa (microzooplankton) when there is an insufficient supply of sterols in their diet. The second alcohol was (22*R*)-30a,30b-dihomohopan-30b-ol (**XXII**), thought to be a transformation product of bacteriohopanetetrol (**XXI**) which is a cell membrane rigidifier found in a number of aerobic bacteria including cyanobacteria, which were abundant in the lake.

The method of production of these two components is unclear. It is not known if the tetrahymanol ester is produced by ciliates themselves in a process analogous to the production of the cholest-5-en-3β-ol (**A1**) SCE by copepods when grazing on a diet which does not contain cholesterol, by contribution of cholest-5-en-3β-ol from their

own bodies. This is considered unlikely given that there have not been any reports of the production of highly non-polar chl transformation products by ciliates (e.g. Strom, 1993). The second possibility is that this component is produced by macrozooplankton grazing on a mixed diet of both ciliates and algae.

The origin of the second component is also unknown. As stated above the esterified alcohol is thought to originate from bacteriohopanetetrol; however, (22*R*)-30α,30β-dihomohopan-30β-ol (**XXII**) has not been found in organisms so it appears that the conversion of bacteriohopanetetrol occurs in the water column. This situation is complicated by the fact that cyanobacteria are generally consumed by microzooplankton which do not appear to produce in large quantities the transformation product (i.e. pyrophaeophorbide *a*; Strom, 1993 and above) needed to form the triterpenoid chlorin esters. An alternative is that this product is produced during grazing of cyanobacteria by small crustaceans such as smaller copepods and copepod nauplii. Cyanobacteria have previously been shown to be present and undigested in copepod faecal pellets (Silver and Aldredge, 1981; Johnson *et al.*, 1982) so this route perhaps does not seem likely as cyanobacteria are picoplankton (0.2-2.0 μm), which are probably too small to be suitable prey for copepods.

6.2.2. Present Study

As a first preliminary step in attempting to elucidate the pathway for the production of triterpenoid chlorin esters, samples of a natural community of mesozooplankton (200-500 μm; comprising mainly small crustaceans and copepod nauplii) was isolated from seawater (Plymouth Sound, U.K.) and allowed to graze on cultures of a cyanobacterium (*Synechococcus* sp. strain DC_2) which were either fresh, had been stored at 4°C overnight immediately prior to feeding, or had been frozen overnight immediately prior to feeding in order to try to induce senescence in a preliminary attempt to promote conversion of bacteriohopanetetrol to (22*R*)-30a,30b-dihomohopan-30b-ol (**XXII**). After 24 h additional aliquots of culture were added to the feeding beakers. Samples of the culture (t=0 and t=24 h) and total particulates (animals, culture and other material) were collected and analysed by HPLC-MS.

6.3. RESULTS: *O. MARINA* AND GREEN ALGA

6.3.1. Green Alga

The green alga pigment distribution (fig. 6-3) is dominated by both C-13^2 epimers of chl *a* (4 and 4', **I**) as well as the mono-oxygenated allomer 13^2-OH chl *a* (peak 12, **XII**). Also present are both C-13^2 epimers of chl *b* (3 and 3', **II**) as well as C-15^1 OH chl *b* lactone (10, **XXXI**). Minor chlorin components include both C-13^2 epimers of phaeophytin *a* (8 and 8', **VIII**), hydroxyphaeophytin *a* (6 and 6', **XIII**) and 13^2-hydroxychlorophyllone *a* (29 and 29', **XVI**). A number of carotenoids were also present which were not investigated further. Peak 38 is discussed below.

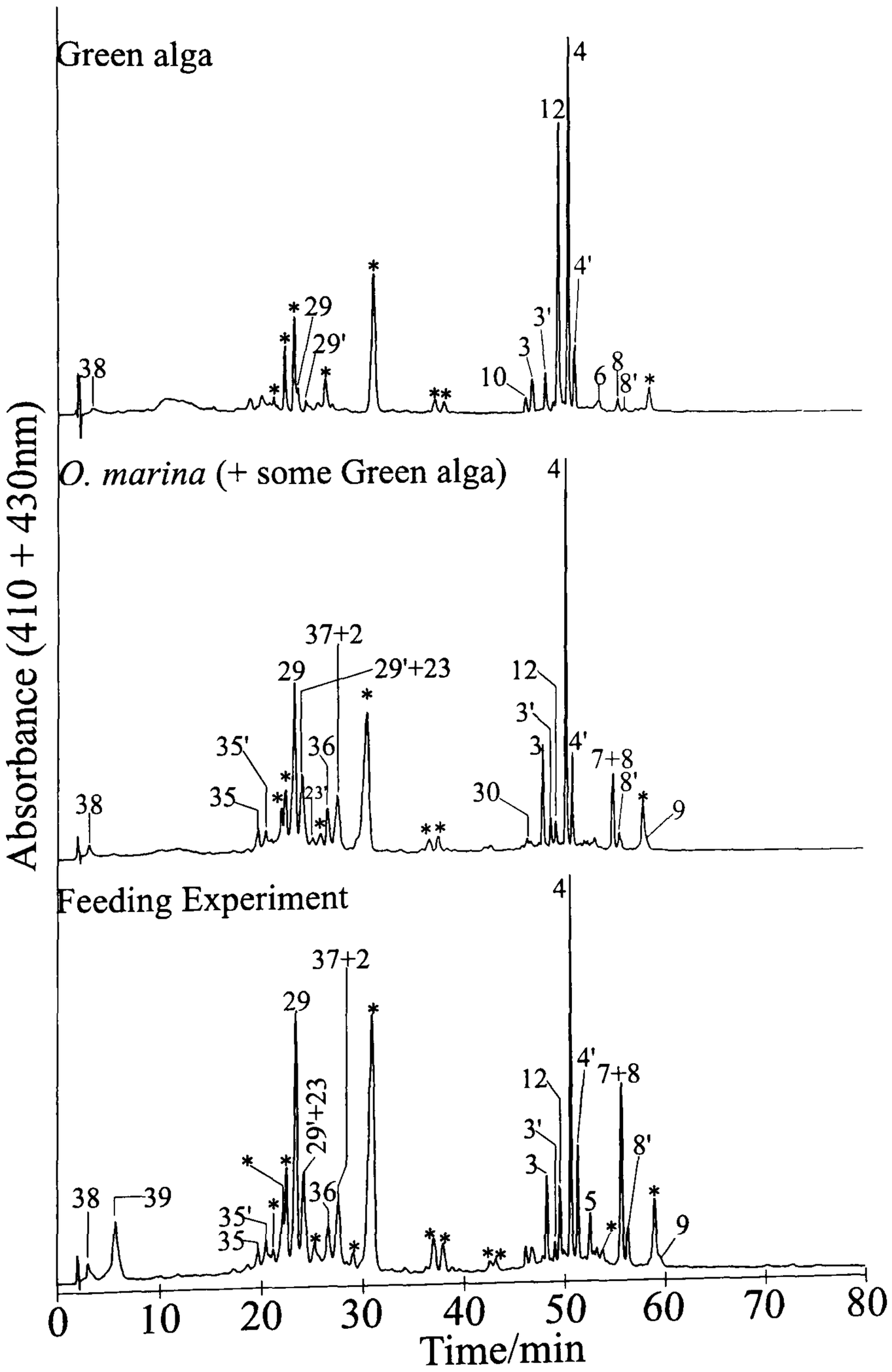


Figure 6-3. *HPLC chromatograms (400+430 nm) from O. marina and green alga experiment (* indicates carotenoid).*

6.3.2. *O. marina* (+ some green alga cells)

The sample containing mostly *O. marina* cells is also dominated by both C-13^2 epimers of chl *a* (4 and 4') and with a much lower contribution from the mono-oxygenated allomer (12). The two epimers of chl *b* (3 and 3') are also present. Chlorophyll *a* transformation products included phaeophytin *a* (8 and 8'), pyrophaeophytin *a* (9, **XI**), co-eluting with a carotenoid, and pyrophaeophytin *b* (7, **XXIX**) co-eluting with the more abundant epimer of phaeophytin *a* (*cf.* Chapter 2). The polar chlorins are dominated by both epimers of 13^2-hydroxychlorophyllone (29 and 29'). It can be seen from the mass and electronic data (figs. 6-4 and 6-5) that the more abundant peak exists as a single component but the ion at *m/z* 593 in the mass spectrum of peak 29' indicates that this component is co-eluting with phaeophorbide *a* (23, **VII**). This ion is also present in the mass spectrum of peak 23' indicating the phaeophorbide *a* epimer (23', **VII**); however, the spectra of this peak are poorly resolved due to the low abundance of the component and co-elution with a carotenoid.

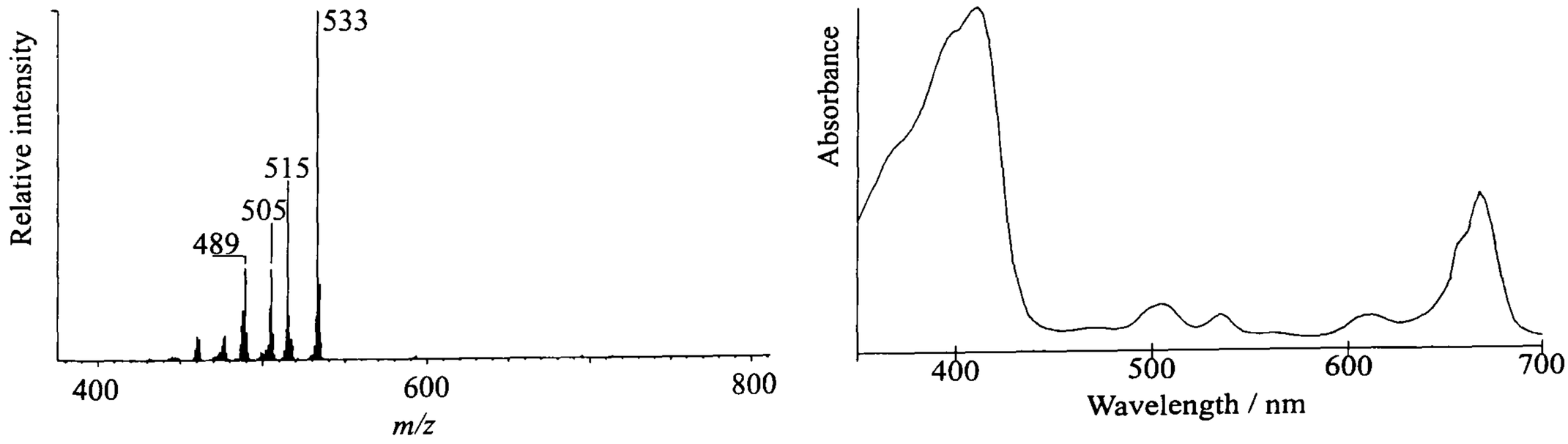


Figure 6-4. *Mass and electronic spectra of peak 29 (13^2-hydroxychlorophyllone a)*

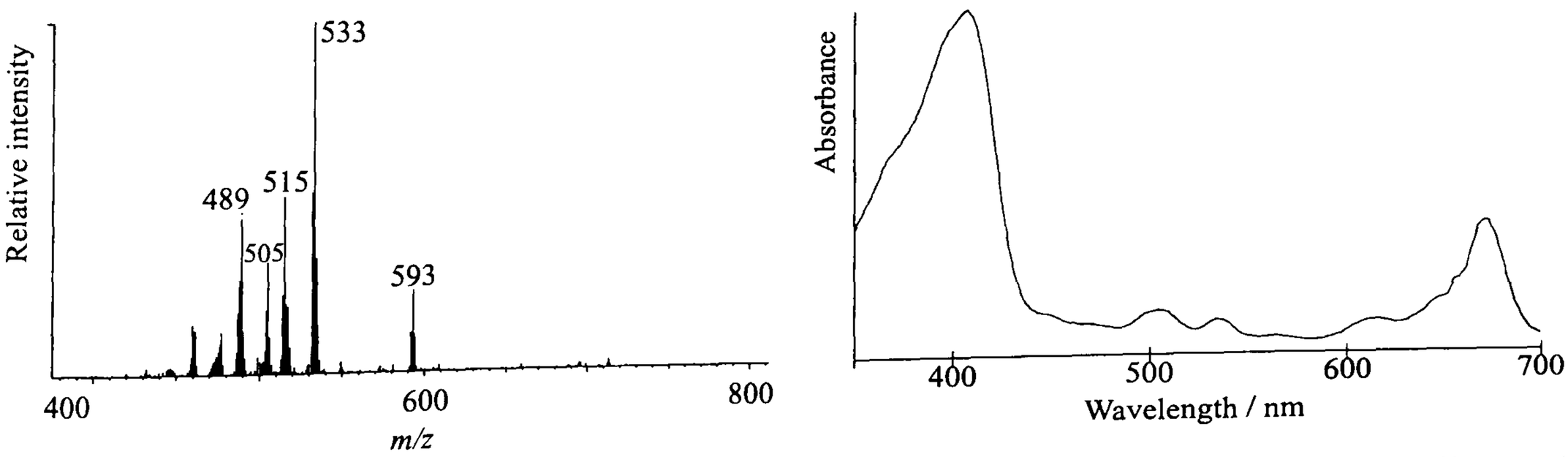


Figure 6-5. *Mass and electronic spectra of peak 29'+23 (13^2-hydroxychlorophyllone a' and phaeophorbide a).*

Two minor peaks (35 and 35') eluting prior to chlorophyllone *a* have electronic spectra (fig. 6-6) similar to that of pyrophaeophytin *b* ($\lambda_{max.}$ 434, 599, and 654 nm; *cf.* Chapter 2). The mass spectrum of peak 35 (fig. 6-6) has ions at *m/z* 547 (MH^+), 529 and 519, directly analagous to *m/z* 533 (MH^+), 515 and 505 in the spectrum of chlorophyllone *a* (see above and *cf.* Chapter 4). Therefore peak 35 is assigned as 13^2-hydroxychlorophyllone *b* (**XXXXVIII**). The mass spectrum of peak 35' is unclear and no diagnostic ions can be recognised; however, given the similarity in the electronic spectrum and the relative retention times this peak is tentatively assigned as as 13^2-hydroxychlorophyllone *b* epimer (**XXXXVIII**; see Table 6-1 for summary of data for novel components with respect to protozoan herbivory).

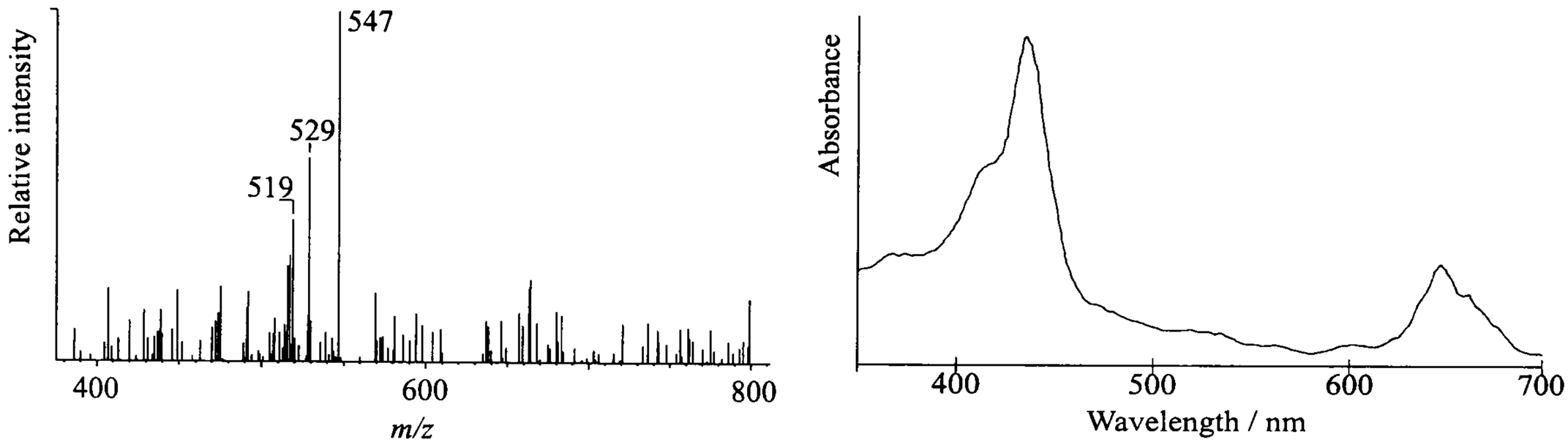


Figure 6-6. *Mass and electronic spectra of peak 35 (13^2-hydroxychlorophyllone b).*

Peak*	MH^+	other ions	$\lambda_{max.}$/nm	Assignment	Structure
30	517		356, 422, 623, 683	$13^2,17^3$-cyclophaeophorbide *a* enol	**XXXXIV**
35	547	529, 519	434, 599, 654	*13^2-OH chlorophyllone b*	**XXXXVIII**
35'	no.	no.	434, 599, 654	*13^2-OH chlorophyllone b epimer*	**XXXXVIII**
36	549	521,503, (624, 475, 425, 407)	386, 416, 512, 677	*13^2-oxopyrophaeophorbide*	**XXXXIIIa**
37	565	(535)	410, 545, 698	purpurin-18	**XIVb**
38	no.	no.	467, 602, 653	chl *b*-like	
39	no.	no.	416, 614, 665	chl *a*-like	

*see fig. 6-3; brackets indicates ion present but not from assigned component; assignments in italics tentative; no. = not observed.

Table 6-1. *Pigment data for noteworthy components in O. marina and green alga experiment.*

Peak 36 has an electronic spectrum ($\lambda_{max.}$ 386, 416, 512 and 677 nm; fig. 6-7) similar to that of 13^2-oxopyrophaeophytin *a* (**XXXXIIIb**; *cf.* peak 27, Chapters 4 and 5). The mass spectrum has ions at *m/z* 549 (MH^+), 521 and 503 which are also present in the spectrum

of 13^2-oxopyrophaeophytin *a* and indicate the 13^2-oxopyrophaeophorbide *a* (**XXXXIIIa**) nucleus. Given the position at which it elutes (just prior to pyrophaeophorbide *a*, see below) it seems likely that this component is 13^2-oxopyrophaeophorbide *a*. The other ions observed in the mass spectrum are thought to be due to co-elution.

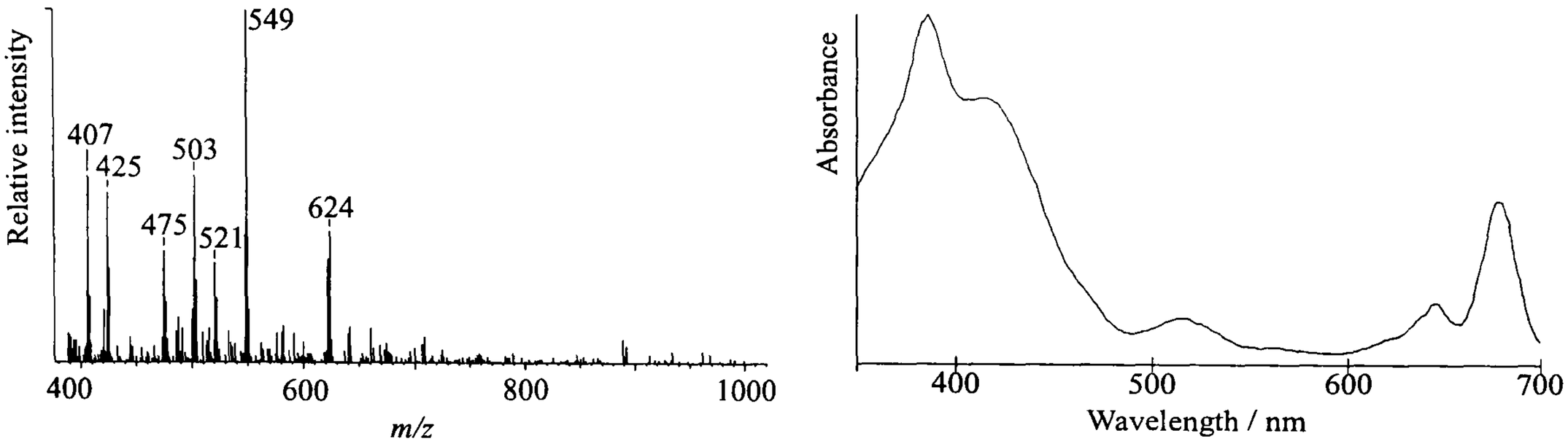


Figure 6-7. *Mass and electronic spectra of peak 36 (13^2-oxopyrophaeophorbide a).*

The mass and electronic spectra (fig. 6-8) of peak (37 plus 2) show characteristics of two components. The electronic spectrum taken from the leading edge of the peak shows purpurin characteristics ($\lambda_{max.}$ 410, 545 and 698 nm; fig. 6-8a) and the electronic spectrum from the trailing edge of the peak ($\lambda_{max.}$ 410, 503, 533, 608 and 665 nm; fig. 6-8b) is similar to that of pyrophaeophorbide *a*. The mass spectrum (fig. 6-8c) has MH^+ at *m/z* 565 indicating purpurin-18 (37, **XVIb**) and MH^+ at *m/z* 535 indicating pyrophaeophorbide *a* (2, **X**).

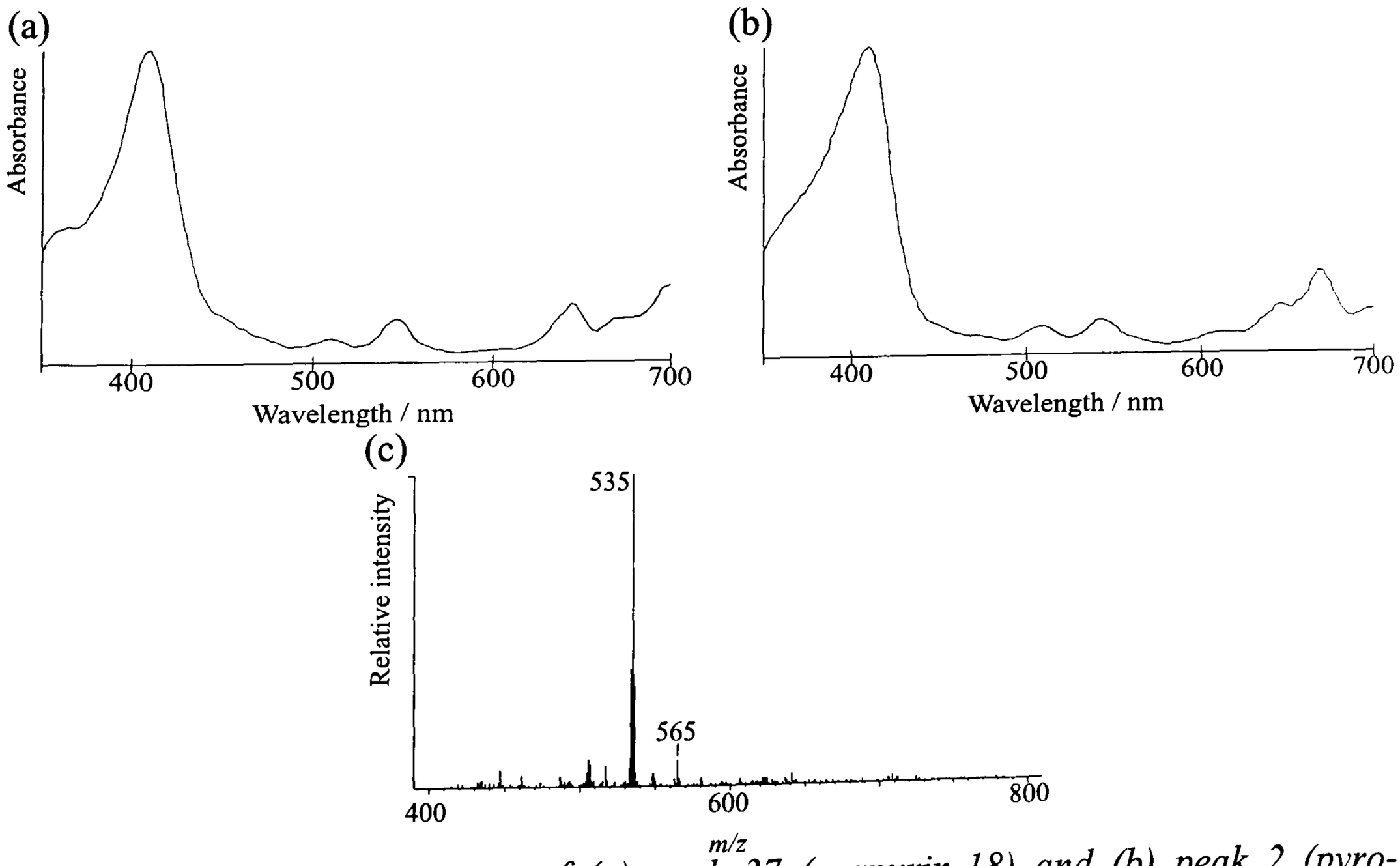


Figure 6-8. *Electronic spectra of (a) peak 37 (purpurin-18) and (b) peak 2 (pyro-phaeophorbide a) and mass spectrum of (c) peaks 37 and 2.*

Also present as a minor component is 13^2,17^3-cyclophaeophorbide *a* enol (30, **XXXXIV**; *cf.* Chapter 5), peak 38 (see below) and a number of carotenoids which were not investigated further.

6.3.3. Feeding Experiment

The distribution (fig. 6-3) is essentially the same as that of the *O. marina* sample except for some minor changes in the relative abundance of some of the major components. Most notable is the increase in the abundance of both epimers of phaeophytin *a* and chlorophyllone *a* relative to chl *a* and the absence of peak 30 (13^2,17^3-cyclophaeophorbide *a* enol, **XXXXIV**). Peak 5 is assigned as phaeophytin *b* (**XVIII**). Also present are two highly polar components peaks 38 and 39 which are only apparent in the electronic data. The electronic data for peak 38 ($\lambda_{max.}$ 467, 602 and 653 nm; fig. 6-9a) suggests a chl *b*-related component and that for peak 39 ($\lambda_{max.}$ 416, 614 and 665 nm; fig. 6-9b) suggests a chl *a*-related component.

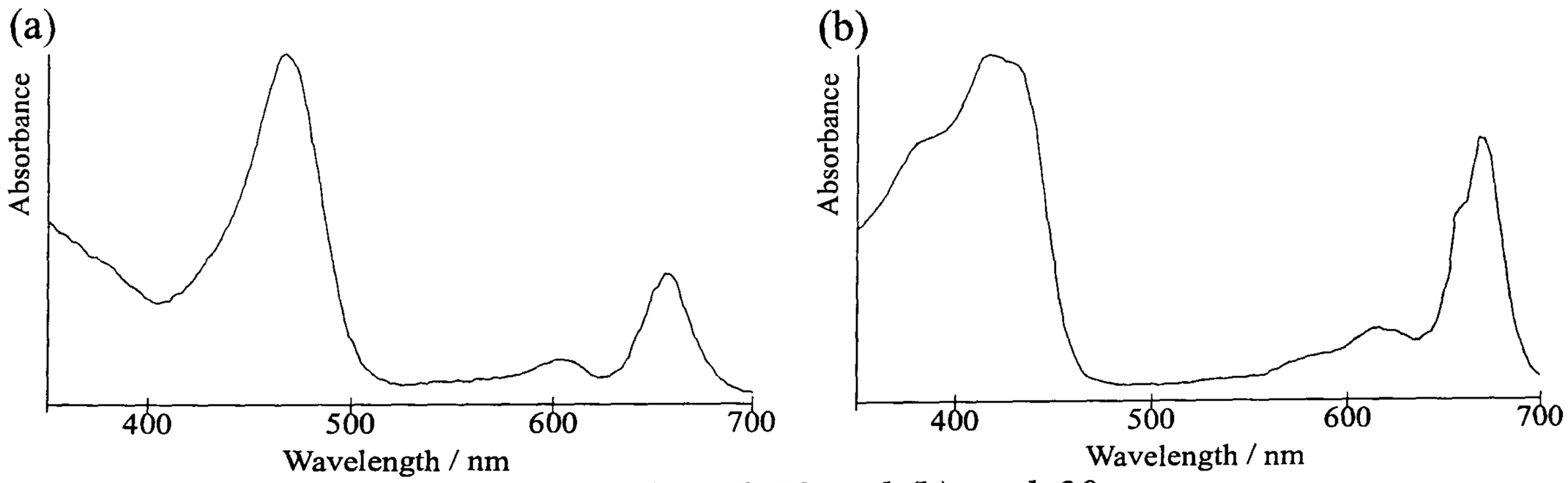


Figure 6-9. *Electronic spectra of (a) peak 38 and (b) peak 39.*

6.4. RESULTS: *O. MARINA* AND *I. GALBANA*

6.4.1. Culture

The distribution (fig. 6-10) is similar to that found in the copepod and *I. galbana* experiment (Chapter 3), being dominated by both epimers of chl *a* (4 and 4', **I**) as well as the mono and di-oxygenated allomers of chl *a* 13^2-OH chl *a* (peak 12, **XII**) and 15^1-OH chl *a* lactone (peak 12, **XXX**) along with minor contributions from both C-13^2

epimers of phaeophytin *a* (8 and 8', **VIII**) and hydroxyphaeophytin *a* (6 and 6', **XIII**) as well as a number of carotenoids which were not investigated further.

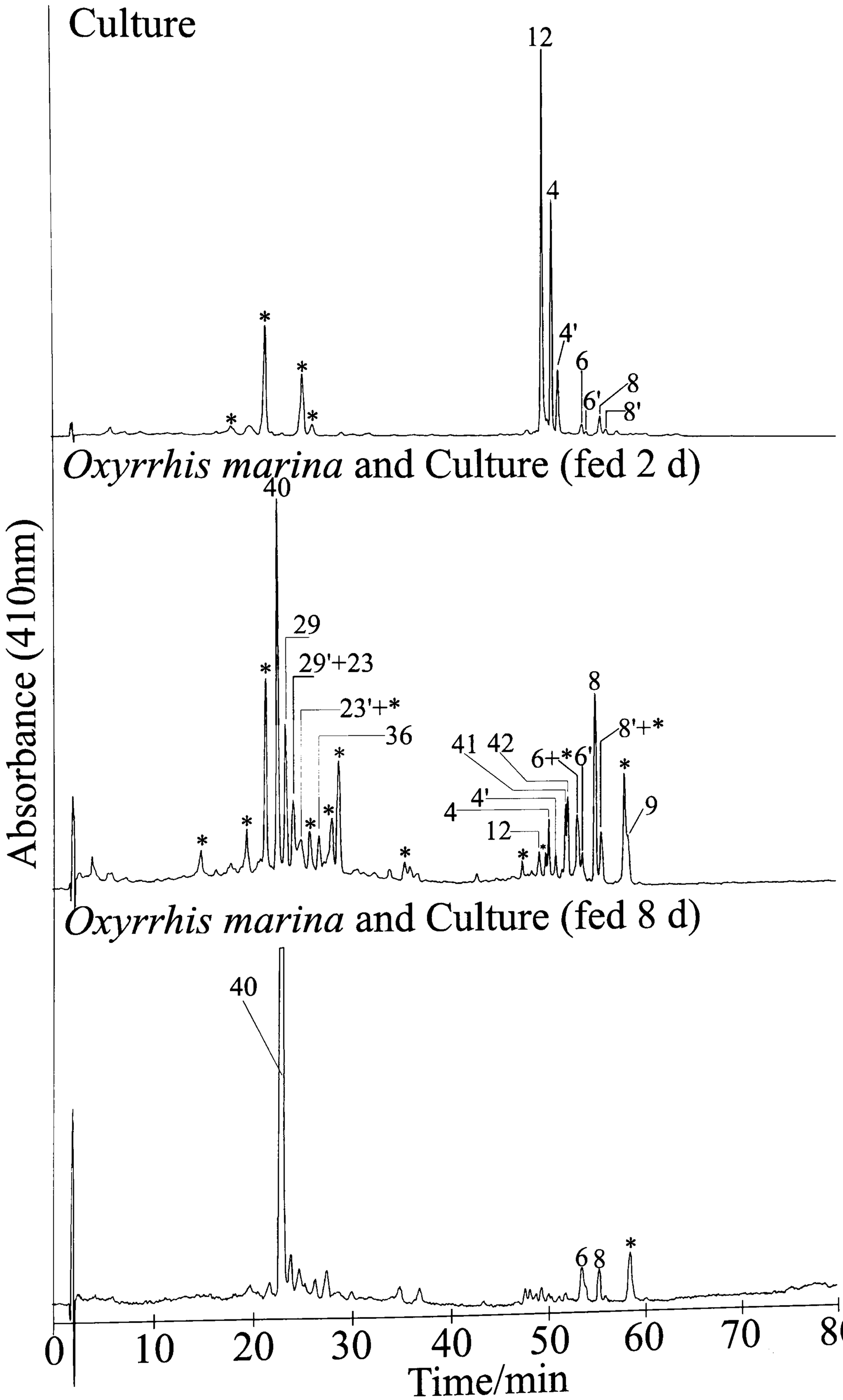


Figure 6-10. *HPLC chromatograms (400 nm) from O. marina and I. galbana experiment (* indicates carotenoid).*

6.4.2. Feeding Experiment (fed 2 d prior to harvesting)

The pigment distribution is dominated by an unknown component, peak 40, which was only observed in the electronic data. It is not clear if this component is a carotenoid as the electronic spectrum ($\lambda_{max.}$ 383 and 641 nm; fig. 6-11) is unusual with the absorbance maxima showing a significant hypsochromic shift from the normal region of maximum absorbance for the more commonly observed algal carotenoids components (i.e. *ca.* 430-480 nm).

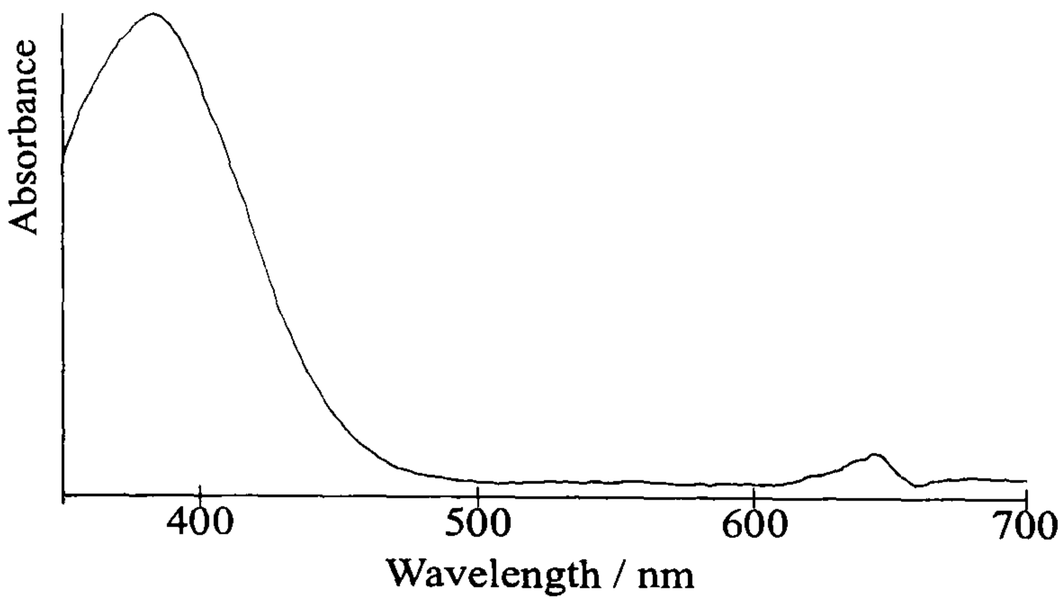


Figure 6-11. *Electronic spectrum of peak 40.*

The chlorin components present include both C-13^2 epimers of phaeophytin *a* (8 and 8', **VIII**), hydroxyphaeophytin *a* (6 and 6'. **XIII**) and hydroxychlorophyllone *a* (29 and 29', **XVI**), with minor contributions from chl *a* (4 and 4'), 13^2-OH chl *a* (12), pyrophaeophytin *a* (9) again co-eluting with a carotenoid (see above), phaeophorbide *a* (23 and 23') and peak 36 assigned as 13^2-oxopyrophaeophorbide *a* (**XXXXIIIa**; see above). Surprisingly, there was no indication of pyrophaeophorbide *a* (**X**). Purpurin-18 (**XIVb**) was also absent. Also present are a pair of partially co-eluting peaks (41 and 42) between chl *a*' and 13^2-hydroxyphaeophytin *a*. Although the mass spectrum (fig. 6-12), with MH^+ at *m/z* 903, and ions at *m/z* 886 (loss of OH), *m/z* 625 and 609 (loss of $C_{20}H_{38}$ from *m/z* 903 and 887[?] respectively) and *m/z* 549 (loss of the C-13^3 carbomethoxy group from *m/z* 609) is similar to that of 13^2-hydroxyphaeophytin *a* lactone (**XXXIII**), this is not indicated in the electronic spectra of either peak ($\lambda_{max.}$ 407, 533, 644 and 665 nm; fig. 6-12) which do not show the expected hypsochromic shift of the Soret band associated with lactones (*cf.* Chapter 2); therefore the structures of these chl *a* transformation products could not be assigned. The ions at *m/z* 709 and 655 are thought to be due to the presence of a carotenoid co-eluting with peak 42.

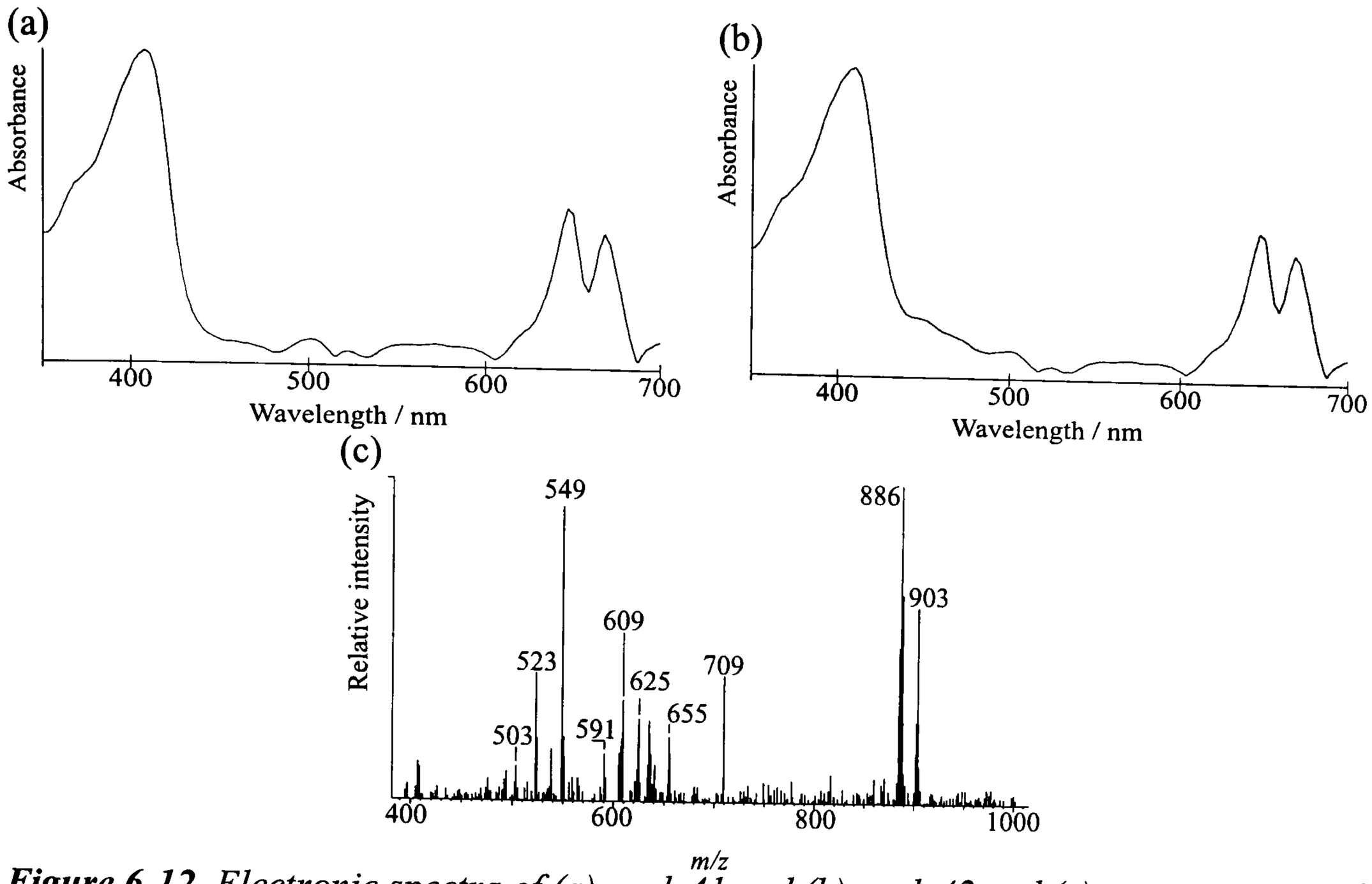


Figure 6-12. *Electronic spectra of (a) peak 41 and (b) peak 42 and (c) mass spectrum of combined peaks.*

6.4.3. Feeding Experiment (fed 8 d prior to harvesting)

The distribution contained only a single abundant component, peak 40 (see above) with trace contributions from a number of chlorins and carotenoids only two of which could be identified: phaeophytin *a* (peak 8) and hydroxyphaeophytin *a* (peak 6).

6.5. DISCUSSION: MICROZOOPLANKTON EXPERIMENTS

Two experiments in which the heterotrophic dinoflagellate *O. marina* was allowed to graze on either an uncharacterised chlorophyte or the haptophyte *I. galbana* failed to demonstrate the production of SCEs, suggesting the possibility that they may not be a product of herbivory by heterotrophic dinoflagellates. Unfortunately, in her experiment with the heterotrophic dinoflagellate *Gymnodinium* sp. grazing on either the green alga *micromona pusilla* and the haptophyte *I. galbana* or the cyanobacterium *Synechococcus* sp. strain DC_2 and *I. galbana*, Strom (1993) did not continue her HPLC analysis after the elution of pyrophaeophytin *a* (phaeophytin a_3) so it is not possible to say if SCEs were produced in that experiment or not. The present experiments did, however, demonstrate some surprising results, particularly in the light of two earlier reports which

stated that *O. marina* produced only minute amounts of chl degradation products (Klein *et al.*, 1986) or none at all (Barlow *et al.*, 1988).

The results have demonstrated the production of not only the components currently seen as representative of macrozoplankton grazing (phaeophytin [**VIII**], pyrophaeophytin [**XI**], phaeophorbide [**VII**] and pyrophaeophorbide [**X**]; e.g. Head and Harris, 1992) but also a number of other components not previously associated with microzooplankton grazing . The most abundant of these components in both the green alga and haptophyte experiments were 13^2-hydroxychlorophyllone *a* (chlorophyllone, **XVI**; e.g. Sakata *et al.*, 1990; Harris *et al.*, 1995a) and its C-13^2 epimer. This component is not considered to be produced during herbivory as it has been found in a number of diatom mono cultures (e.g. Sakata *et al.*, 1990, 1994). Therefore two possible explanations in this case for the occurrence of these components in the products of these experiments are proposed. They may have been present in the culture only in trace abundance and were concentrated up in the feeding experiment total particulates due to their apparent greater stability relative to other chlorins (see chapter 4), the majority of which may have been converted directly to colourless products (e.g. Klein *et al.* 1986; Barlow *et al.* 1988). The second possibility is that the difference in the mode of ingestion (i.e. phagotrophy) compared with crustaceans and in digestive conditions may activate endogenous algal enzymes which produce this product, possibly providing a source of antioxidants for the protozoa as proposed for invertebrates (e.g. Watanabe *et al.*, 1993; Sakata *et al.*, 1991, 1994). The presence of a minor amount of these components in the algal culture in the first experiment may be due to the presence of a minor contribution to the fraction from detrital particles, e.g. fragmented *O. marina* cells or faecal material, which were collected along with the green alga fraction. Microscopic examination of this fraction after separation did not reveal the presence of any intact *O. marina* cells. This suggests therefore that the chlorophyllone was probably contained within the heterotroph as this organism has previously been shown to produce only non-pigmented faecal material in the form of empty algal cysts and also is known to ingest cells at a faster rate than that at which it degrades chl (Barlow *et al.*, 1989). The occurrence of two abundant phaeophorbide *a*-like peaks (termed phaeophorbides a_4 and a_5; Strom, 1993) was observed in all of the products of a series of feeding experiments involving 5 species of

ciliate and one heterotrophic dinoflagellate and a variety of different algal substrate species including haptophytes, dinoflagellates, a chlorophyte and a cyanobacterium. Strom (1993) proposed that these two products could be diagnostic of protozoan herbivory; however, assuming that they are the same products as observed here i.e. the two C-13^2 epimers of chlorophyllone, this would not be possible as they are known to also be associated with a number of other sources, particularly diatoms (see Chapters 4 and 5). The presence of 13^2-hydroxychlorophyllone *b* (**XXXXVIII**) is the first report of this novel component and represents a previously unrecognised pathway for the biotransformation of chl *b* and by analogy with chlorophyllone *a* probably also acts as an antioxidant.

Two other novel components (13^2-oxopyrophaeophorbide *a* [**XXXXIIIa**] and purpurin-18 [**XIVb**]) were also present in the green alga experiment and the former was also found in the haptophyte experiment. These components have also been suggested to been found as antioxidants in a number of marine invertebrates (Watanabe *et al.*, 1993; Sakata *et al.*, 1994). Interestingly, pyrophaeophorbide *a* (**X**) was also absent from the haptophyte experiment although its precursors pyrophaeophytin *a* (**XI**) and phaeophorbide *a* were present, suggesting less complete digestion of this organism at the stage at which the sample was harvested (i.e. 2 days after addition of a fresh culture aliquot; *cf.* Strom, 1993).This could also indicate that the production of chlorins represents only a minor pathway for chl *a* transformation in this organism (*cf.* Daley, 1973).

In contrast to the above results, the sample from the *I. galbana* experiment which had been last fed at 8 days prior to harvesting showed an almost total absence of any pigmented material except for the single unknown component (see above). This is in agreement with the results of Klein *et al.* (1986) and Barlow *et al.* (1988) for the same organism, who demonstrated total removal of all pigments within 50 h. However, the green alga experiment represents a more stable situation in which the alga was able to reproduce at a rate sufficient to sustain the increasing numbers of the herbivore cells with the culture being maintained simply by addition of aliquots of culture media containing the nutrients required by the alga, which could be likened to a bloom

situation. High abundance of chlorophyllone in the presence of a consistent food source (i.e. green alga experiment) could explain in part (see also Chapter 4) the high concentration of chlorophyllone (assigned previously as MW532 pyrophaeophorbide by King, 1995) found in sediment traps in the Black Sea, with this component showing concentration maxima coinciding with the occurrence of the spring and autumn blooms (King, 1995).

An outline of the pathways of chl *a* biotransformation demonstrated in these feeding experiments is shown in Figure 6-13.

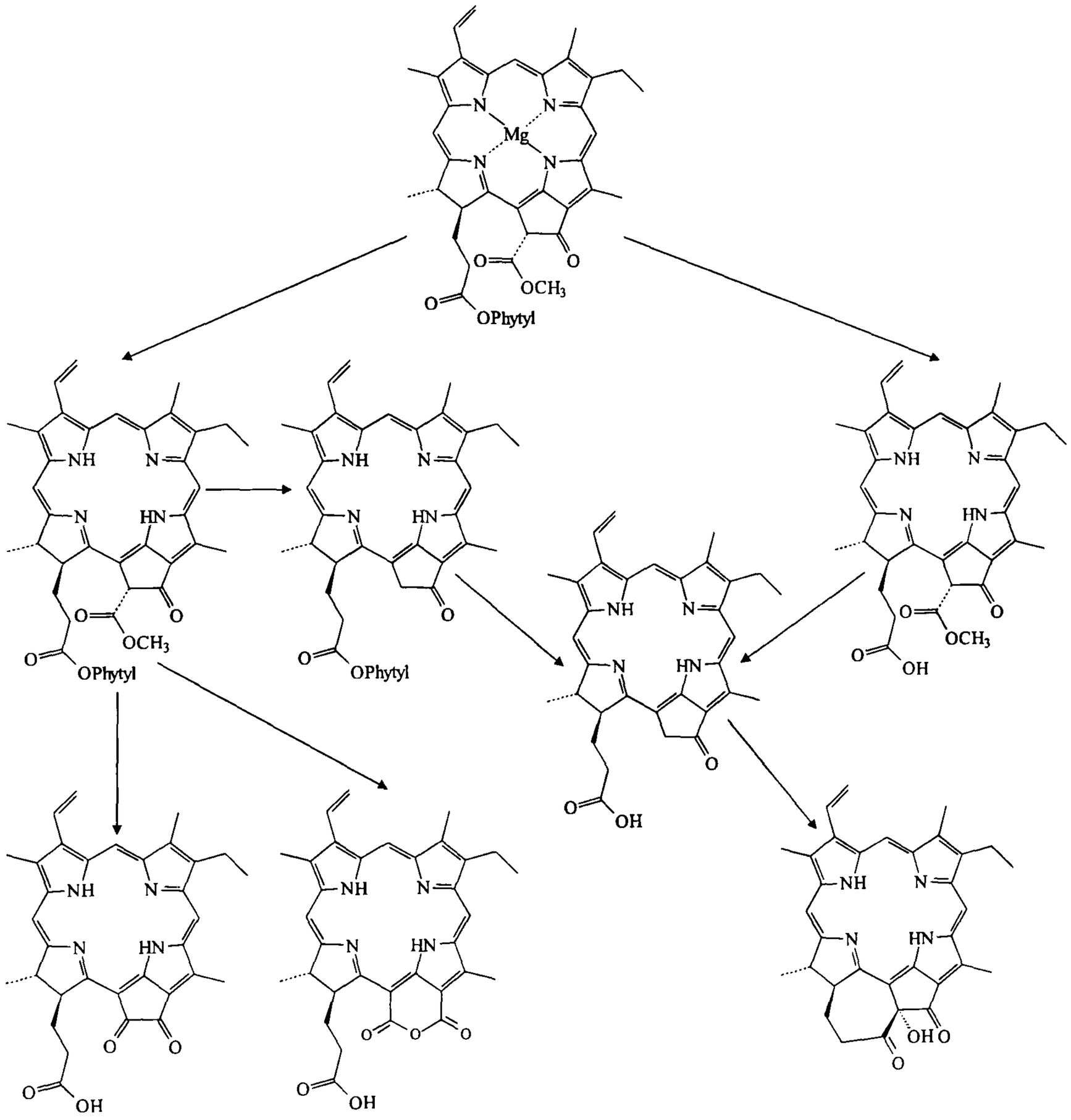


Figure 6-13. *Transformation pathway for chl a operating in O. marina feeding experiments.*

Interestingly, both purpurin-18 and 13^2-oxopyrophaeophorbide *a* were found in the dephytylated form, in contrast to the copepod experiments where only their phytylated counterparts were observed. This suggests that a different transformation pathway is operating in this situation, resulting in rapid loss of the phytyl group. This is also

supported by the low relative abundance of pyrophaeophytin *a* in these experiments compared to the copepod experiments where it was the most abundant individual product in all but one case. If SCEs are produced by transesterification of pyrophaeophytin *a* rather than by esterification of pyrophaeophorbide *a* then their absence in the products of protozoan herbivory could be a result of the apparent faster rate of dephytylation operating in protozoa relative to crustaceans. Conversely, it also seems likely that the production of pyrophaeophorbide *a* is relatively uncommon for protozoa as Strom (1993) did not detect it in the products of feeding experiments with six different species of protozoa. Hence, if SCEs are produced by esterification of pyrophaeophorbide *a* they may not be observed in protozoa due to the low abundance of the pyrophaeophorbide *a* component. It is not possible to ascertain if SCEs could have been present in the products of the protozoa feeding experiments conducted by Strom (1993) as the chromatograms shown terminate almost immediately after the elution of pyrophaeophytin *a* (assigned phaeophytin a_3).

6.6. RESULTS: MESOZOOPLANKTON EXPERIMENT

6.6.1. Fresh Culture (0 h)

The distribution (fig. 6-14) is dominated by chl *a* and its C-13^2 epimer (4 and 4', **I**) and 13^2-OH chl *a* (12, **XII**). There is no indication of other chl *a* transformation products. A number of carotenoids are also present but were not investigated further. The distributions of the two cultures which had been stored at 4° or -20°C were similar except for a small amount of phaeophytin *a* and its C-13^2 epimer (**VIII**).

6.6.2. Fresh Culture (24 h)

After 24 h the fresh culture (fig. 6-14) contains a small proportion of phaeophytin *a* (8, **VIII**) and 13^2-hydroxyphaeophytin *a* (6, **XIII**) as well as the components present in the fresh culture.

6.6.3. Feeding Experiment

The total particulate sample (fig. 6-14) contained the same components as the culture after 24 h with only slight increases in the abundance of phaeophytin *a* and 13^2-hydroxyphaeophytin *a*. No additional chl *a* products were observed and no carotenoid alteration products were apparent.

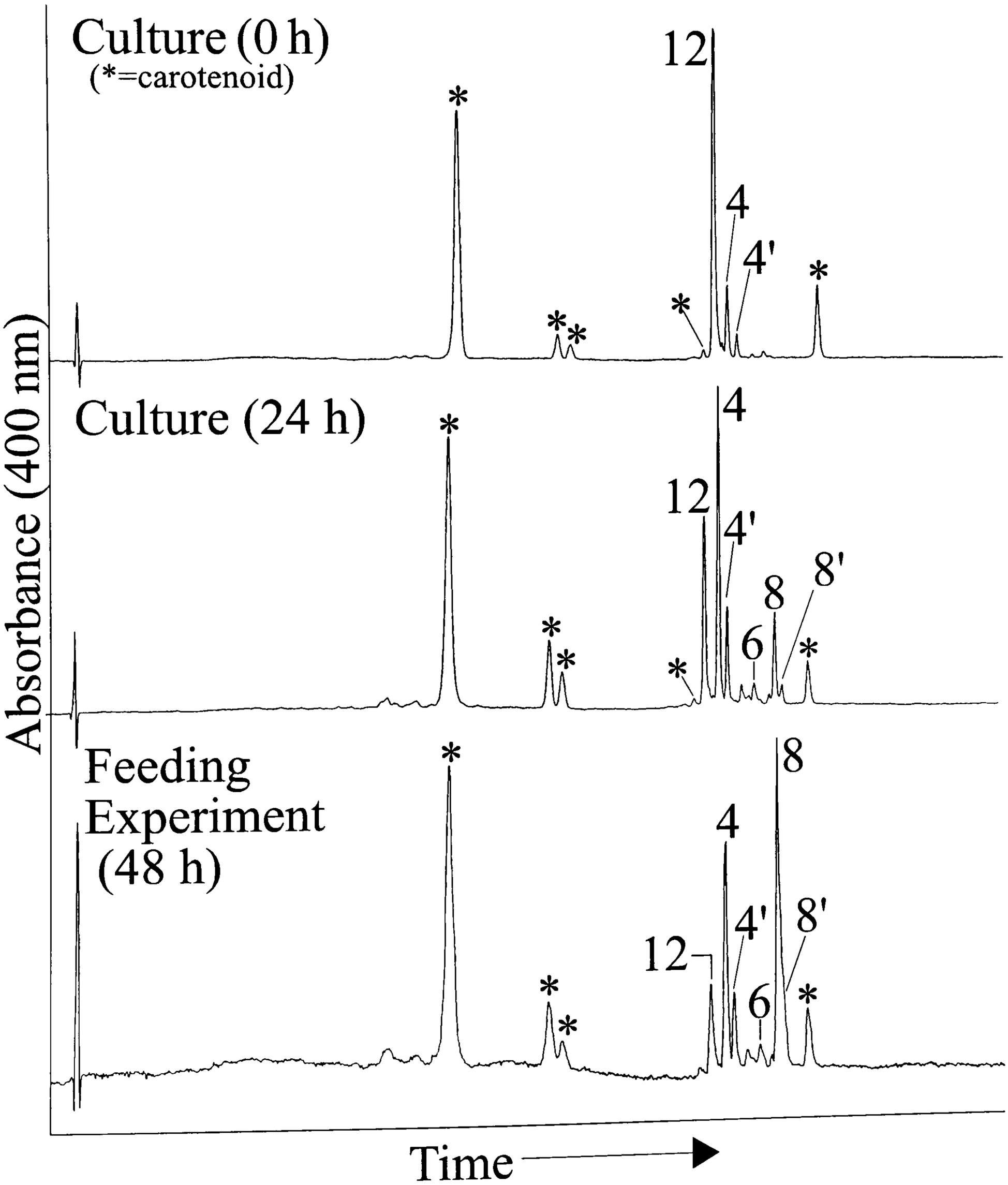


Figure 6-14. *HPLC chromatograms (400 nm) from mesozooplankton (200-500μm) feeding on fresh Synechococcus culture.*

6.7. DISCUSSION: MESOZOOPLANKTON EXPERIMENT

Culture samples of the cyanobacterium *Synechococcus* sp. contained only chlorophyll *a*, its monoxygenated allomer, their phaeophytin counterparts and several carotenoids. When a mixed community of mesozooplankton (200-500 μm) was allowed to graze on this organism there was no indication of the production of any chl *a* biotransformation products except for a slight increase in the abundance of phaeophytin *a* and its allomer which were observed to be produced in the culture alone after 24 h. This strongly suggests either that the *Synechococcus* sp. passed through the animals without being assimilated or that the chl was converted directly to colourless components although it is more likely that the zooplankton community was not able to graze on this organism as it was too small for capture and ingestion.

Therefore, from this experiment there was no evidence to support the idea that direct grazing of cyanobacteria by mesozooplankton is not responsible for the production of triterpenoid chlorin esters such as (22*R*)-30a,30b-dihomohopan-30b-ol pyro phaeophorbide *a* or any of the other common pigment grazing indicators found in the water column and sedimentary environment.

6.8. SUMMARY

Two experiments with the heterotrophic dinoflagellate *O. marina* feeding on either a green alga or a haptophyte (*I. galbana*) did not demonstrate the production of SCEs. However, a surprising number of chl *a* and *b* biotransformation products were observed in the total particulates, including phaeophytin *a* and *b*, pyrophaeophytin *a* and *b*, phaeophorbide *a* and pyrophaeophorbide *a*, 13^2-hydroxychlorophyllone *a*, 13^2-oxopyrophaeophorbide *a* and purpurin-18. Also reported for the first time is the novel component 13^2-hydroxychlorophyllone *b* and its C-13^2 epimer. These findings represent a new source for the production of chlorophyllone, one of the most commonly occurring sedimentary chlorins.

Previous studies with this protozoan have failed to yield chl transformation products in more than trace abundance and it is unclear why they were found in such high abundance in this study. However, the results provide further evidence that microzooplankton are important components in aquatic environments which can, at least under certain conditions, be responsible for significant transformation and/or removal of algal chl.

No triterpenoid chlorin esters were formed by direct grazing of mixed mesozooplankton population on a cyanobacterium (*Synechococcus* sp.), so it is not possible to say if they are produced in this manner. Clearly further studies are required (see Chapter 7).

Chapter 7

GENERAL DISCUSSION AND FUTURE WORK

7.1. GENERAL DISCUSSION

7.1.1. Aims

The main aims of this work were to investigate the following questions:

i. How widespread is the production of SCEs (and related products) in the marine environment?

ii. How accurately do SCE sterol distributions reflect those of their precursor sterols (algal and animal)?

iii. How stable are SCEs and their sterol distributions during ageing?

iv. What other chl transformation products are commonly produced during herbivory?

7.1.2. Formation of Steryl Chlorin Esters

The preliminary study of Harradine *et al.* (1996b) in which the SCEs were observed to be products of diatom grazing by a copepod has been extended to include species from 3 of the other major algal divisions (Chlorophyta, Haptophyta, Dinophyta) and a more detailed study of a diatom species. SCEs were observed in the faecal pellets produced by the copepod *Calanus helgolandicus* grazing on individual cultures of algae from four of the major algal divisions (see Table 7-1.)

Division	Class	Species	Chapter
Chlorophyta (Green algae)	Prasinophyceae	*Tetraselmis suecica*	2
	Chlorophyceae	*Dunaliella tertiolecta*	
	Chlorophyceae	*Chlamydomonas reginae*	
Haptophyta	Prymnesiophyceae	*Pleurochrysis carterae*	3
Bacillariophyta (Diatom)		*Thalassiosira weissflogii*	4
Dinophyta (Dinoflagellates)		*Prorocentrum micans*	5
		Alexandrium tamarensis	

Table 7-1. *Algal species used in experiments which demonstrated the production of SCEs during grazing by the copepod C. helgolandicus.*

A further two species of haptophyte (*Coccolithus pelagicus* and *Isochrysis galbana*) were also investigated, but failed to yield SCEs although they may have been present in

quantities below detection limits. It is thought that they were not observed due to the high assimilation efficiency of the smaller non-coccoid species (*I. galbana*) from which very little faecal material was produced. In the case of the larger, heavily armoured species (*C. pelagicus*), although pellet production was high, the pellets consisted mainly of carbonate liths and so had a very low organic carbon content. These findings will be of particular importance when investigating regions known to have abundant haptophyte populations, particularly tropical and subtropical oceans, as use of SCE sterol distributions could lead to significant underestimation of the contribution of haptophytes to the overall phytoplankton population (see also Chapter 3).

Where SCEs were produced, their proportion of the total chlorins in the faecal pellets was variable (table 7-2), both immediately after grazing as well as over a period of ageing up to *ca.* 30 d (discussed in more detail below).

Algal Species	SCE % total chlorins (0 d)	SCE % total chlorins (*ca.* 30 d)
P. carterae	55	na
*T. weissflogii**	8.5	16
*P. micans**	17	59

na = no ageing. * = large scale experiment

Table 7-2. *SCEs as % of total chlorins in faecal pellets.*

Products with properties similar to those of SCEs (Downs, 1989) and later suggested to be SCEs (King and Repeta, 1994) have also been observed in the faecal pellets of euphausiids, *Pleuroncodes* crabs, *Phialidium* medusea and *Pleurobrachia* (Downs, 1989), and have also been found in the guts and faecal pellets of salps. They are not, however, ubiquitous products of grazing as they were not observed in the total products when the heterotrophic dinoflagellate *Oxyrrhis marina* grazed on two different algae (*I. galbana* and a green alga; Chapter 6) and have not been observed during other experiments with similar organisms (e.g. Barlow, *et al.*, 1988; Strom, 1993). This suggests that SCEs can not be used as general indicators of herbivory by all zooplankton size classes.

Although SCEs have now been shown to be produced from a variety of algal species, it is still not clear whether they are formed by transesterification of pyrophaeophytin *a* or esterification of pyrophaeophorbide *a*. Where SCEs were observed, both of the two chlorin components were also present and in all but one case (small scale *P. micans*; Chapter 5) pyrophaeophytin *a* was more abundant than pyrophaeophorbide *a*, which might argue in favour of transesterification. On the other hand, the low abundance of pyrophaeophorbide *a* may be a result of its esterification.

7.1.3. Comparison of SCE and Substrate Sterol Distributions

King and Repeta (1991) first suggested that sedimentary SCE sterol distributions might reflect more closely than sedimentary free sterols the distribution of free sterols in the upper zones of the corresponding water column. It was therefore proposed that sedimentary SCEs could provide a more accurate indicator of the sterol distributions originally biosynthesised in the photic zone of the water column and hence would be more accurate indicators of the original phytoplankton community structure. Further evidence to support this suggestion has been observed during studies of a number of marine and lacustrine environments (e.g. Eckardt *et al.*, 1992; Pearce *et al.*, 1998). The preliminary feeding experiment study (Harradine *et al.*, 1996b) also supported this hypothesis by showing a good correlation between the algal sterol distribution and SCE sterol distributions after the animal-derived sterol cholesterol was excluded from the comparison. This was also supported by the good correlation between the sterol distribution of the suspended particulate matter and the SCEs produced during grazing of this material by salps in the Sargasso Sea (King and Wakeham, 1996).

In order to examine more closely the relationship between SCE and substrate free sterol distributions the sterol distributions from five different algal species were determined and compared to those of the SCEs derived from them during grazing by *C. helgolandicus*. These experiments highlighted a number of factors which can lead to alteration of both the algal and animal substrate sterol distributions prior to esterification (see below).

7.1.3.1. Algal Cholesterol Content

Background

The most abundant sterol in most crustaceans, including copepods, is cholesterol (**A1**). It is accepted that these animals are incapable of *de novo* sterol biosynthesis (Goad, 1978, 1981) so they must acquire it either directly from their diet or by conversion of other phytosterols, particularly C-24 alkylated sterols, to cholesta-5,24-dien-3β-ol (desmosterol, **A2**) and ultimately to cholesterol (see fig. 2-46; Chapter 2). Previous studies have shown that specific sterols are taken up by copepods more readily than others; for example Harvey *et al.* (1987) found that sterols with unsaturation at either $\Delta^{8(14)}$ or $\Delta^{17(20)}$ were the main source of cholesterol for *C. helgolandicus* when feeding on the dinoflagellate *Scrippsiella trochoidea* which contains at least 25 sterols, with cholesterol being only a minor component. Bradshaw *et al.* (1989) found that crustaceans seemed to convert C_{28} sterols to cholesterol more readily than C_{29} sterols. Conversely, stanols and ring saturated stenols appear to be passed through the copepod gut with little or no uptake or alteration (Harvey *et al.*, 1987, 1989). Sterols with a Δ^7 double bond are also thought to be less readily assimilated than those with a Δ^5 or $\Delta^{5,7}$ unsaturation, possibly indicating that crustaceans lack the enzyme required for the $\Delta^7 \rightarrow \Delta^{5,7}$ conversion (Corner *et al.*, 1986).

This study

The prasinophyte *T. suecica* was found to contain only two C_{28} sterols (24-methylcholesta-5,24(28)-dien-3β-ol [**A4**] and 24-methylcholest-5-dien-3β-ol [**A5**]). These sterols were incorporated into SCEs in the same relative abundance to that in which they occurred in the alga (Chapter 2). This suggests three possibilities: (i) both sterols were taken up for conversion to cholesterol with equal efficiency; however, this seems unlikely given that the monounsaturated component (Δ^5) must first be converted to the diunsaturated form ($\Delta^{5,24(28)}$) as part of the assimilation pathway (see fig. 2-46); (ii) esterification to pyrophaeophorbide *a* (**X**) occurs more rapidly than the dehydrogenation and dealkylation reactions which again seems unlikely given the results of other experiments in which the algal sterol distribution shows significant alteration (see below) and (iii) the sterols were not utilised in this experiment. Some evidence supporting the third possibility is apparent from the reduction in the abundance of

cholesta-5,24-dien-3β-ol in the fed animals relative to the starved animals. Whatever the reason the SCE sterol distribution directly reflected that of the source organism.

The haptophyte *P. carterae* contained a more complex sterol distribution consisting of five sterol components including 24-methylcholesta-5,22-dien-3β-ol (**A6**), 23,24-dimethylcholesta-5,22-dien-3β-ol (**A10**) and a third C_{29} sterol tentatively identified as 23,24-dimethylcholesta-5,23(28)-dien-3β-ol (**A12**). These sterols were taken up into the SCE fraction; however, a further two unusual components tentatively identified as 4α,23,24-trimethylcholesta-5,23(28)-dien-3β-ol (**D12**) and a minor component 23,24-dimethylcholesta-5,22,25-trien-3β-ol (**A13**) were not present in the SCEs. This is the only experiment during this work in which a major algal sterol components were not clearly observed in the SCE fraction. The reasons for this are unclear but there may have been several contributing factors. The C_{29} trienol was only a minor component in the alga so the SCE derived from it may simply have been below detection limits. The high degree of unsaturation may also be a factor, as to date no examples of SCEs containing trienols have been conclusively identified in sedimentary SCE fractions (Eckardt *et al.*, 1991b, 1992; King and Repeta, 1991, 1994; Chillier and Gülaçar, 1995; King and Wakeham, 1996; Pearce *et al.*, 1998; Laureillard *et al.*, 1997). The lack of the C_{29} trienol SCE may also be important with respect to other species of alga such as the chlorophytes which are commonly found to contain complex sterol distributions with triunsaturated sterols such as $\Delta^{5,7,22}$ sterols. It is also possible, however, that sterols such as this are taken up for conversion to cholesterol more readily than other sterols which would, in this case, suggest that assimilation occurs more readily than the esterification process. The absence of the more abundant diunsaturated 4-methyl sterol from the SCE fraction is also difficult to explain, although again no diunsaturated 4-methyl sterols have previously been reported in sedimentary SCE fractions, which may simply be a result of a low concentration of this type of sterol in the natural environment. These results suggest therefore, that certain unusual sterols are not readily converted to SCEs, suggesting in turn that contributions to the sediment from organisms containing such sterols and those which are most readily assimilated by the herbivore may be underestimated. This would by default indicate enhanced input from other organisms

containing the more commonly observed algal and SCE sterols (i.e. C_{27}-C_{29} Δ^5, $\Delta^{5,22}$ and $\Delta^{5,24(28)}$).

Both the *P. carterae* and *T. weiss* experiments demonstrated significant alteration of the algal sterol distribution prior to esterification. In the haptophyte experiment the abundance of the two C_{29} diunsaturated sterols was significantly reduced in the SCEs relative to the diunsaturated C_{28} sterol. This at first sight disagrees with the results of Bradshaw *et al.* (1989; see above) which suggested that C_{28} sterols would be assimilated more readily than C_{29} sterols. However, in this experiment the two C_{29} sterols had 23,24 substitution so the presence of the carbon at the C-23 position may have affected the rate of dealkylation.

The results from the diatom experiment were complicated by a number of factors, including the presence of some of the algal sterols in the animals at the commencement of feeding. However, it is clear that the majority of the SCE sterols were derived from the alga (fig. 4-20; Chapter 4). Algal sterols other than 24-methylcholesta-5,24(28)-dien-3β-ol were present as SCEs in higher abundance relative to this sterol compared to the algal culture, which suggests enhanced uptake of this major C_{28} sterol relative to the C_{29} sterols. In this case this is in agreement with the general conclusion of Bradshaw *et al.* (1989) and also indicates that assimilation of sterols for conversion to cholesterol occurs more rapidly than esterification to pyrophaeophorbide *a* (see also below).

Cholesterol was either absent from the culture (haptophyte) or present in only trace abundance (diatom) in the above experiments. As a consequence the sterol distributions underwent alteration prior to esterification. On the other hand, in the small scale *P. micans* (dinoflagellate) experiment cholesterol was the most abundant algal sterol and was a major component of the culture in the large scale experiment. In both of these studies the relative distributions of the other 4-desmethyl sterols (excluding the 24-ethylcholest-5-en-3β-ol SCE which co-elutes with a 4-methyl sterol SCE) were comparable with that of the original culture. This suggests that when cholesterol is present in the diet in quantities sufficient for the animal's needs, the SCE 4-desmethyl

sterol distribution (excluding animal-derived sterols) will be directly representative of the algal source excluding trienols (see above).

The incorporation of animal sterols including cholesterol into the SCE fraction was clearly observed in the prasinophyte, haptophyte and diatom experiments and was also suggested by mass chromatography analysis in the large scale dinoflagellate experiment. In each experiment the three animal sterols (cholesterol, cholesta-5,24-dien-3β-ol, **A2** and cholesta-5,22-dien-3β-ol, **A3**) were present in the starved animals prior to the commencement of feeding. In the experiments with the two algae which did not contain cholesterol (prasinophyte and haptophyte), the $\Delta^{5,24}$ sterol desmosterol (**A2**) was present in the SCE fraction in higher abundance relative to cholesta-5,22-dien-3β-ol than in the animals. This observation provides further support to the suggestion that sterol assimilation commences prior to esterification to pyrophaeophorbide, since desmosterol is an intermediate in the pathway for the conversion of C-24 alkylated phytosterols to cholesterol. In the haptophyte experiment cholesterol was also present in the SCEs in significantly higher abundance relative to cholesta-5,22-dien-3β-ol (fig. 3-19; Chapter 3), suggesting a high degree of assimilation of sterols from this alga (*P. carterae*); this may explain at least in part the absence of two of the algal sterols from the SCE fraction (see above). The situation in the large scale *T. weiss* experiment is more complicated as cholesterol was also present in the alga, although only in trace abundance (< 2% total algal sterols). Again desmosterol was present in the SCEs in higher abundance relative to cholesta-5,22-dien-3β-ol (fig. 4-20; Chapter 4) with the abundance of cholesterol being comparable to that in the animal (*cf.* prasinophyte experiment; Chapter 2), in agreement with the apparent uptake, particularly of 24-methylcholesta-5,24(28)-dien-3β-ol, during this experiment (see above).

In summary, it can be clearly seen that the presence and abundance of cholesterol in the algal diet of the copepod and the lability of the other phytosterols for conversion to cholesterol are key factors in the processes governing the alteration (if any; e.g. Chapter 2) of both the algal and animal substrate sterol distributions prior to esterification.

7.1.3.2. 4-Methyl Sterols

The presence of 4-methyl sterols in the algal sterol distribution also led to alteration of the distribution, with 4-methyl sterols being present in the SCEs in significantly reduced abundance relative to that in the substrate. This effect was observed in all three feeding experiments performed with dinoflagellates (*A. tamarensis* and two with *P. micans*) and is attributed to a steric effect of the 4-methyl group interfering with the esterification process. This effect is therefore expected to be apparent in the natural environment (see also below). The results are summarised in Table 7-3.

Experiment	% 4-methyl sterols (Substrate)	% 4-methyl sterols (SCEs)
A. tamarensis	37	22
P. micans (small scale)	44	26
P. micans (large scale)	72	28

Table 7-3. *Abundance of 4-methyl sterols in algal substrates and SCEs.*

Previous workers (King and Repeta, 1991, 1994; Pearce *et al.*, 1998) have demonstrated that in sedimentary SCE fractions, 4-methyl components are present in significantly lower abundance than in the corresponding free sterol fraction. This difference has been attributed to the greater lability of free desmethyl sterols to biodegradation relative to 4-methyl sterols (e.g. Teece, 1994), but can now be seen to be the result of two combined effects i.e. also a result of discrimination against initial formation of 4-methyl SCEs due to the steric effect mentioned above. By default, this would imply that incorporation into SCEs would protect 4-desmethyl sterols from preferential biodegradation. The results from the pellet ageing experiment in the present study with the dinoflagellate (large scale *P. micans*; Chapter 5) provided the first direct evidence for this in that the sterol distribution remained unchanged after 30 days (see below).

7.1.4. Pellet Ageing

Two large scale feeding experiments (*T. weiss*, Chapter 4; *P. micans* Chapter 5) have demonstrated that there was no change in the SCE sterol distribution when faecal pellets were aged in seawater for up to 30 d. This suggests that faecal pellets containing SCEs

could be transported out of the photic zone to a depth of up to 3000 m (sinking rate *ca.* 100 m d^{-1}; Corner *et al.*, 1986) with the SCE sterol distribution still intact.

Although the SCE sterol distribution remains intact during ageing, SCEs were found, however, to undergo significant degradation over the thirty day ageing period, with only *ca.* 30% of the initial mass remaining (fig. 4-22a; Chapter 4). Furthermore, SCEs were observed to be more resistant to this degradation relative to other chlorins, particularly phaeophytin *a*, with their proportion of the total chlorins increasing from *ca.* 8 to 16 % over 30 d in the *T. weiss* experiment (fig. 4-22b; Chapter 4) and from *ca.* 17 to 59% over 29 d in the *P. micans* experiment. Therefore, the greater stability of SCEs relative to other chlorins explains the high abundance of such components in the sedimentary environment.

The pellet free sterol signature over the first 8 d of ageing in the dinoflagellate experiment (Chapter 5) showed that little change had occurred in the relative abundance of the major components; however, after 29 d the greater resistance of free 4-methyl sterols than their desmethyl counterparts to biodegradation was apparent for the two major 4-methyl sterols, providing further evidence that SCE sterol distributions are more stable than free sterol. Further evidence suggesting that SCE distributions are more robust biomarkers is found in the fact that the SCEs were significantly more stable than free sterols over this period. Although the free sterols were initially present in the pellets at an order of magnitude higher than the SCEs, after only 29 d the difference had fallen to just three times greater (fig. 5-32b; Chapter 5).

Comparisons of the SCE and free sterol abundance in a variety of environments has revealed that the relative concentration of these components is highly variable depending on the environment. For example, Pearce *et al.* (1998) found that free sterols were approximately two orders of magnitude higher in abundance in Black Sea surface sediment whilst in the Baltic Sea and in Priest Pot (Cumbria, U.K.), a eutrophic lake, the free sterols were *ca.* one order of magnitude higher than the SCE sterols. However, in the highly eutrophic Lake Valencia (Venezuela) SCE sterols were actually in higher abundance than the free sterols, possibly indicating higher grazing pressure in this

environment, and resulting in the production of larger quantities of SCEs compared to the other locations studied.

7.1.5. Pellet Sterilisation

In order to investigate the method by which SCEs and other chlorins are degraded, two pellet samples from the large scale *P. micans* experiment (Chapter 5) were sterilised with $HgCl_2$ and then aged in the usual way for 29 d. After ageing the chlorin distribution, including the SCEs, was most similar to the fresh pellet sample, suggesting that SCEs are degraded in faecal pellets due to biological activity rather than chemical reactions (i.e. oxidation). Previous studies have shown that copepod faecal pellets contain bacteria from the gut when they are egested and also that they are more rapidly colonised by bacteria on their outer surface when sinking through a water column than in laboratory studies when sinking is not simulated. This would suggest that the measurements of the extent of pigment and sterol degradation found in this work should be seen only as minimum estimates.

7.1.6. Alternative Chlorin Nuclei

To date, pyrophaeophorbide *a* is by far the most abundant chlorin nucleus found in SCEs with only a limited number of exceptions being known (see Chapter 1). In the course of these experiments the production of SCEs containing the pyrophaeophorbide *b* nucleus (SCEs *b*) has been demonstrated directly for the first time in the laboratory (large scale *T. suecica* experiment; Chapter 2). The sterol distribution within the SCE *b* fraction was comparable with that of the SCEs *a*, indicating that chl *b* undergoes the same transformation as chl *a* during herbivory and hence that SCEs *b* should be important components in bottom sediments below water columns containing a high population of chl *b* containing species of phytoplankton. Indeed, in the course of this work such species have been found in Mediterranean sapropels (Cariou-Le Gall *et al.*, 1998) and by the author in the Baltic Sea (Kowalewska *et al.*, 1999).

One other novel SCE-like component was identified in the products from the large scale *T. weiss* experiment and is tentatively identified as 13^2-oxopyrophaeophorbide *a* 24-ethylcholesta-5,24(28)-dien-3β-yl ester (see fig. 4-10; Chapter 4). The chlorin moiety of this component is discussed in more detail below.

No indication of phaeophorbide steryl esters was observed in any of the experiments and may therefore be a reflection of the fact that phaeophorbide *a* was not observed in the pellets from any of them, since there appears to be no *a priori* reason why the phaeophorbide esters cannot be formed (see below). Recently, Goericke *et al.* (1999) have reported the presence of both phaeophorbide *a* and pyrophaeophorbide *a* carotenols (specifically fucoxanthin alteration products) esters in a number of recent sediments. Such components were not observed during the present study even though at least one of the algal species used contained this carotenoid (*T. weiss*). Goericke *et al.* (1999) suggested that phaeophorbide sterol esters were not formed due to steric hindrance from the C-13^2 carbomethoxy group which would interfere with the enzymatic esterification process with bulky substrates such as sterols, whereas in the case of the carotenoids which have a more elongated structure this steric effect may not be as apparent. However, studies by Pearce (1994) have shown that in a purely chemical reaction esterification of sterols to either phaeophorbide *a* or pyrophaeophorbide *a* proceeds with equal efficiency. In addition, Riffé-Chalard *et al.* (1999) have recently reported the occurrence of phaeophorbide *a* steryl esters in the oxic bottom sediment of a small eutrophic lake. This first identification of this type of SCE was ascribed to enhanced preservation of the esters in this oxic environment. This seems unlikely, however, as organic matter is generally less well preserved in oxic environments. It seems more likely that their production is indicative of either specific herbivore species or algal species lacking the necessary enzymes required to perform the decarbomethoxylation, i.e. where phaeophorbide is a herbivory product in significant abundance then the corresponding SCEs should be present.

7.1.7. SCE Sterol Distributions *vs.* Free Sterol Distributions

This work has demonstrated that a number of factors can lead to alteration of the substrate sterol distributions prior to esterification to form SCEs. A highly significant factor is the presence and abundance of cholesterol directly available to the herbivore from the phytoplankton diet. When there is low cholesterol in the diet high cholesterol demand leads to significant alteration of the distribution of other sterols due to assimilation and when cholesterol availability is high, low cholesterol demand from other sterol sources means that the substrate sterol distribution is present in the SCE in comparable relative abundance. As a consequence of the above effect, the lability of other available phytosterols to assimilation resulting in conversion to cholesterol will also determine the pattern of alteration observed in the SCE sterol distribution. These factors will of course affect both the free and SCE sterol distributions and abundance in pellets although further studies would be required before it would be possible to say if one fraction is affected more than the other. Certain unusual sterols (e.g. trienols; *cf.* Chapter 3) would appear not to be incorporated into SCEs but if this is again due to cholesterol demand then this factor would again also be observed in the free sterols.

Another factor which alters the pellet SCE distribution is the presence of 4-methyl sterols, which are discriminated against during production of SCEs, and conversely, have been shown to be highly abundant in the free form in faecal pellets and subsequently increase in abundance over time due to their enhanced stability to biodegradation (see above). For example, the common ring saturated 4-methyl sterol dinosterol, has been found to show quantitative passage through the copepod gut (Harvey *et al.*, 1989).

SCEs have been shown to be degraded more slowly than free sterols and their sterol distributions to be more resistant to degradation than free sterol in pellets (large scale *P. micans* experiment; Chapter 5). This indicates that esterification to the chlorin nucleus protects 4-desmethyl sterols from preferential degradation during ageing. Free sterol distributions, however, can show clear alteration after a period of only 30 d clearly demonstrating that SCE sterol are more robust markers of phytoplankton populations.

The above observations suggest overall that SCEs are better markers than free sterols. however, given for example that they were not apparent in all of the haptophyte experiments and would appear to be formed in varying proportion relative to the total chlorins, they do not appear to be good quantitative indicators. Rather, they appear to be more effective as indicators of changes in phytoplankton community structure as demonstrated in particular looking for changes in the phytoplankton community as demonstrated by King and Repeta (1994) in a series of three consecutive sedimentary units in the Black Sea, which showed a high abundance of 4-methyl sterol SCE components in the second unit relative to units 1 and 3 in which the distribution was dominated by desmethyl components. Furthermore, a minor desmethyl sterol component which might not be observed in a sedimentary free sterol fraction due to biodegradation would be expected to be seen in the SCE sterols after hydrolysis or reduction.

7.1.8. Other Chlorin Products

A wide variety of other chlorophyll transformation products were observed, some of which have not previously been reported in feeding experiment products; their occurrence is summarised in Table 7-4. The occurrence, possible transformation pathways and potential significance are discussed below.

7.1.8.1. Allomers

Allomerization is the term used to describe reactions in which the C-13^2 H-atom in chls (or phaeophytins) is replaced by an oxygen-containing moiety. This process can occur by both chemical and enzymatic pathways and as it can occur under both light and dark conditions it is thought to involve oxidation by triplet oxygen ($^3O_2$; i.e. autoxidation) rather than singlet oxygen ($^1O_2$) as light is required to form this species (i.e. photoxidation). Allomerization has been implicated as an early stage step in the breakdown of chl in the natural environment (Brown *et al.*, 1991), leading to the formation of a wide variety of alteration products including ultimately the aetioporphyrins (Woolley *et al.*, 1998 and references therein; and see below). The structural alterations occurring during allomerization are centred around the exocyclic

ring E and the initial steps involved in the proposed reaction mechanism are shown in Figure 7-1.

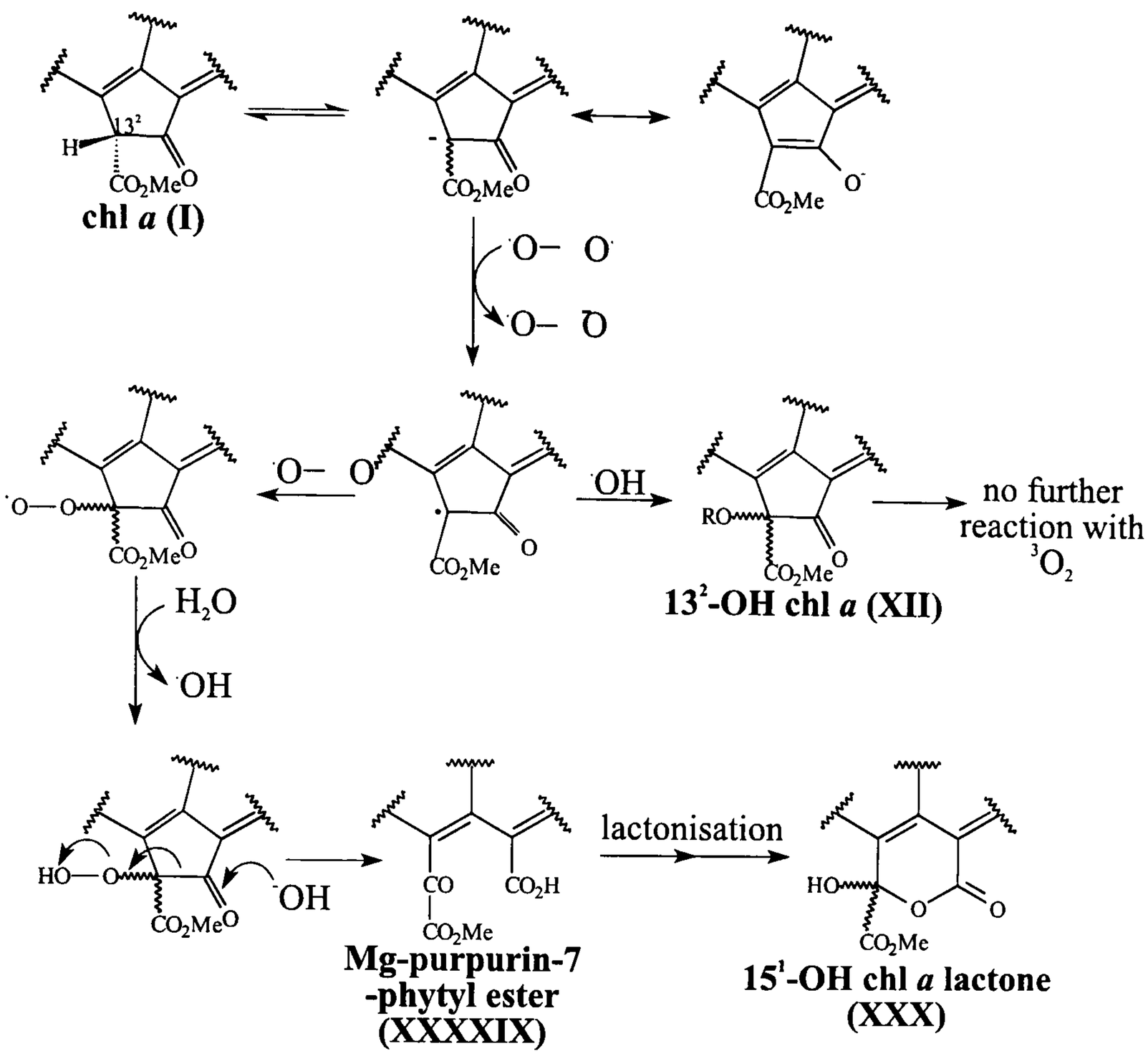


Figure 7-1. *Mechanism of chl a allomerization proposed by Hynninen (1991); adapted from Woolley et al. (1998); for brevity, only ring E is shown.*

This mechanism is of course also valid for reactions with chl *b* and with phaeophytins *a* or *b* and also for reactions involving MeOH, ·OMe and ⁻OMe, resulting in the formation of methoxy allomers (e.g. 15¹-OMe phaeophytin *a* lactone, **XXXIV**). It can be seen that initial formation of the mono oxygenated allomer (e.g. **XII**, fig. 7-1) prevents further reaction with triplet oxygen as the allomer no longer contains an acidic H at C-13², so loss of this species must occur *via* an alternative mechanism to reaction with $^3O_2$. Reaction with a second molecule of $^3O_2$ can occur, however, eventually leading to formation of the ring open purpurin-7 species (e.g. **XXXXIX**, fig. 7-1) which can then undergo lactonisation to form the dioxygenated allomer (e.g. **XXX**, fig. 7-1).

Experiment	*C. helgolandicus* and					*O. marina* and	
Pigment	*T. Suecica**	*P. carterae*	*T.weiss**	*P. micans**	*A. tamarensis*	Green alga	*I. galbana*
Phaeophytin *a*	m[1,2]	m[1]	m[1]	m[1]	M[1]	M[1,2]	M[1]
Pyrophaeophytin *a*	M[2]	M	M	M	M	m[2]	m
pyrophaeophorbide *a*	m[2]	m	t	M	m	m	
SCEs	M[2]	M	m	M	t		
13[2]-hydroxychlorophyllone *a*			t[3]		t[4]	M[2]	M
purpurin-18-phytyl ester	t	t	t[5]	t	m[4]		
pyrpurin-18						m	
13[2]-oxopyrophaeophytin a			t	t	t[4]		
13[2]-oxopyrophaeophorbide a						m	m
Peak 21	t	t					
Peak 22	m	m	m				

* large scale experiment only. M = major component, m = minor component, t = trace component; assignments in italics tentative.
[1] allomers also present; [2] chl *b* counterpart also present; [3] present in aged pellets only; [4] also present in algal control; [5] also present in culture and control.

Table 7-4. *Summary of chl transformation products detected in feeding experiment products.*

7.1.8.2. Purpurins

Purpurins are chl transformation products of Type I oxidation reactions in which the structure of ring E is modified. They are again thought to originate *via* allomerization reactions such as those shown above (e.g. **XXXXIX**, fig. 7-1). Purpurin-18 contains a cyclic six-membered anhydride instead of the original exocyclic ring E. This component and its phytylated counterpart purpurin-18-phytyl ester (**XIVa**) have been found in sedimentary organic matter (e.g. Baker and Louda, 1986; Naylor and Keely, 1998), as antioxidants in a clam (Watanabe *et al.*, 1993), in senescent algal cultures (Louda *et al.*, 1998) and water column particulates (Naylor and Keely, 1998). This indicates that they are formed at the earliest stages of chl degradation, probably during cellular senescence, including cellular disruption resulting from herbivory (Naylor and Keely, 1998).

Such components are important as they have been considered to be indicators of the redox potential of depositional environments (Ocampo and Repeta, 1999) and to be precursors of sedimentary aetioporphyrins (e.g. Baker and Louda, 1986). Related ring opened structures such as Mg-purpurin-7-phytyl ester (**XXXXIX**) and chlorin p_6 (**XV**) are also considered to be intermediates in the formation of aetioporphyrins in which the exocyclic ring E is absent. A typical pathway showing key intermediates in the formation of aetioporphyrins from chl *a* is shown in Figure 7-2 (*cf.* Naylor and Keely, 1998).

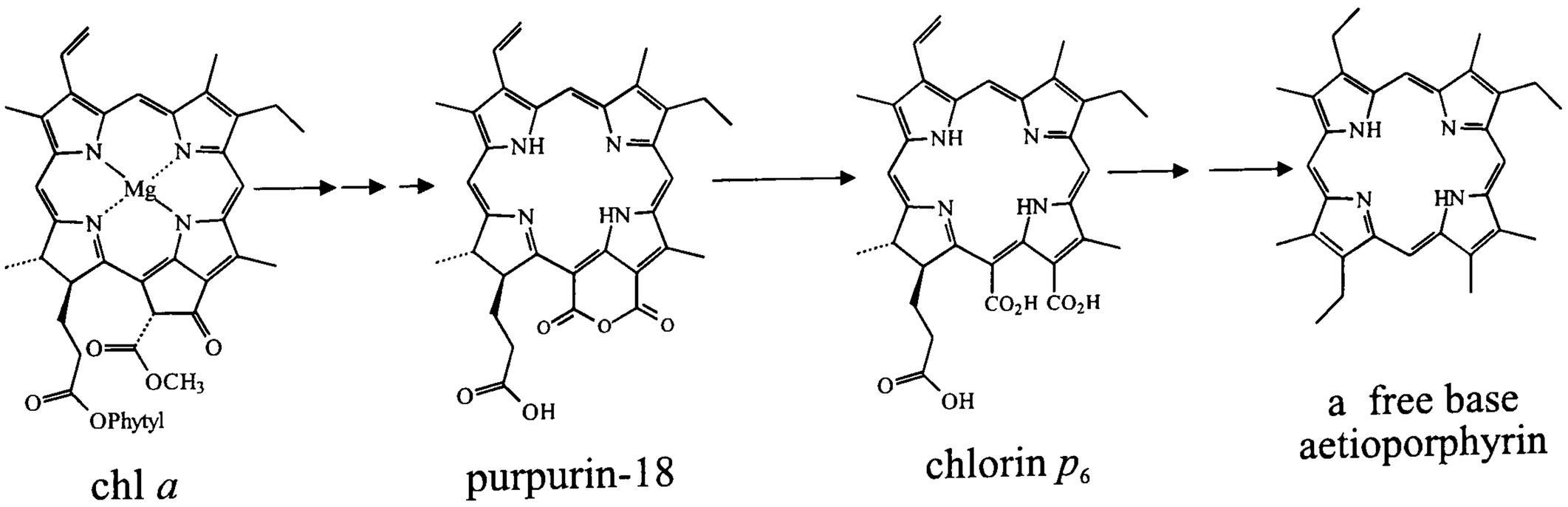


Figure 7-2. *Conversion of chl a to an aetioporphyrin.*

The occurrence of purpurin-18-phytyl ester in the pellets in five of the copepod feeding experiments (see Table 7-4 for algal species) indicates that such products can be produced by or from members of four of the major algal divisions. This component was detected in the culture in the large scale *T. weiss* experiment and in the control in the *A.*

tamarensis experiment. This suggests that it was probably present in trace abundance in all of the algal species used (possibly acting as an antioxidant; *cf.* Watanabe *et al.*, 1993) and was observed in the pellets due to a concentrating effect of the grazing process. Such a concentrating effect may explain why such components have been thought to be indicators of the redox conditions in sedimentary environments. Also of importance is the fact that the presence of such components in faecal pellets may provide an efficient method of transport of these components out of the photic zone to the sediment, enhancing the amount of such components which reach the sediments.

Unlike the copepod experiments, purpurin-18-phytyl ester was not present in the products of the protozoan experiments; however, its dephytylated counterpart purpurin-18, was present, suggesting either more extensive hydrolysis or an alternative transformation pathway to that operating in crustaceans. Disregarding the SCEs, there was a general lower abundance of dephytylated products in all of the copepod experiments compared to the protozoan experiments, indicating a more rapid hydrolysis reaction during protozoan grazing.

7.1.8.3. 13²-Hydroxychlorophyllone a

The occurrence of 13^2-hydroxychlorophyllone *a* (chlorophyllone *a*) in the products of the large scale diatom experiment is perhaps not surprising given that it has previously been detected in trace abundance in a number of diatom species and also in several marine invertebrates known to have been feeding on diatoms, where it was suggested to act as an antioxidant (Sakata *et al.*, 1990, 1994; Watanabe *et al.*, 1993). In addition, prior to the present its occurrence in a sediment rich in diatom frustules was tentatively ascribed to a diatom origin (Keely *et al.*, 1994); its occurrence in a senescent dinoflagellate culture and the faecal pellets derived from it indicate, however, that it is not a specific diatom marker.

Surprisingly, chlorophyllone *a* was also present in significant abundance in the total products when the heterotrophic dinoflagellate *O. marina* grazed either on an unknown green alga or the haptophyte *I. galbana* (Chapter 6). This is the first direct identification of this component as a result of protozoan herbivory, although products with similar

characteristics (assigned phaeophorbide a_4 and a_5) were detected in the products of a series of feeding experiments performed by Strom (1993), involving 6 different protozoan species feeding on a variety of algal species. Based on the abundance of these two components in the products of all of the experiments in the Strom (1993) study it was suggested that these products could be used as indicators of protozoan herbivory (but see below) although the same components had also been identified in the faecal material of various salps and a crab (Nelson, 1989; Downs, 1989). Although these two organisms are quite distinct from herbivorous protozoa it is possible that they may in fact digest pigmented detritus produced by protozoan grazers as suggested by Downs (1989). This is particularly likely in the case of the salps which feed using a mucous net which is capable of retaining very small detrital particles. As salps are macrozooplankton they produce large, rapidly sinking faecal pellets which are transported out of the photic zone with most of their pigment content still intact (Caron *et al.*, 1989), so it is possible that this could be an important mechanism responsible in part for the high abundance of chlorophyllone found in recent and ancient sediments (e.g. Chillier *et al.*, 1993; Harris *et al.*, 1995b; Harris and Maxwell, unpublished results). Also, the pellet ageing studies (diatom experiment) in which this component was evident only in the aged pellets suggests that it is more stable relative to other chlorins, which will also contribute to its high abundance in the sedimentary environment.

A novel component 13^2-hydroxychlorophyllone *b* and its epimer were also detected in the products of the *O. marina* and green alga experiment; this not only represents the first known occurrence of this transformation product but also indicates that chl *b* also undergoes the same rearrangement transformation pathway as chl *a*.

Chlorophyllone is thought to be produced from chl *a* *via* the intermediates pyrophaeophorbide *a* and $13^2,17^3$-cyclophaeophorbide *a* enol (Watanabe *et al.*, 1993; Ocampo *et al.*, 1999; and see fig. 4-25; Chapter 4). The latter of these two intermediates was observed in the senescent algal control in the *A. tamarensis* experiment (Chapter 5) along with chlorophyllone *a*, although it was absent from the faecal pellets, and was also a trace component observed in the heterotrophic dinoflagellate - green alga experiment (Chapter 6).

Given the variety of sources for chlorophyllone *a* (and chlorophyllone *b*) it is clear that this component can no longer be seen a biomarker specific to diatoms (*cf.* Keely *et al.*, 1994), neither can it be used as a marker of protozoan herbivory (*cf.* Strom, 1993). However, it is an important component in the complex transformation pathways undergone by chl *a* in the water column as well as being of interest as a precursor for a number of other chlorins and porphyrins with bicyclic ring systems found in ancient sediments (see Chapter 4).

7.1.8.4. 13^2-Oxopyrophaeophorbide a

Another novel component, 13^2-oxopyrophaeophytin *a* (**XXXXIIIb**), was also observed in the faecal pellets from three of the copepod feeding experiments (see Table 7-4). Prior to this work only the dephytylated counterpart, 13^2-oxopyrophaeophorbide *a* (**XXXXIIIa**), had been observed in a clam which had been feeding on diatoms (Watanabe *et al.*, 1993) and it was therefore suggested to originate from the diatoms or from chl *a* from the diatoms produced by metabolic conversion upon ingestion. The presence of the phytylated component in the algal control and pellets in the *A. tamarensis* experiment has demonstrated that this chlorin moiety is derived not only from diatoms but also from dinoflagellates and that it can be produced during senescence of an algal culture in the absence of grazers, therby precluding its use as a grazing indicator (see below).

Interestingly, as for the purpurin-18 components, only the dephytylated component 13^2-oxopyrophaeophorbide *a* was present as a minor component in the total products of both of the protozoan feeding experiments, again suggesting more rapid hydrolysis during microzooplankton grazing, at least under the conditions used for these experiments (see below).

Watanabe *et al.* (1993) suggested that 13^2-oxopyrophaeophorbide *a* was produced from chlorophyllone *a*. However, the occurrence of the phytylated component precludes this route. Although the mechanism of its formation is unknown, it again seems likely that it is another component resulting initially from allomerization reactions with triplet oxygen (*cf.* fig. 7-1).

7.1.8.5. Formation of Oxygenated Products in Copepod and Protozoan Experiments

Previous reports of the occurrence of oxygenated chl transformation products such as purpurin-18 and 13^2-oxopyrophaeophorbide *a* in trace abundance in cultures of marine diatoms in the absence of grazers (Watanabe *et al.*, 1993; Sakata *et al.*, 1994) indicate that such components are regular algal constituents. This suggests therefore that the presence of the related components (purpurin-18-phytyl ester and 13^2-oxopyrophaeophorbide *a*) in the pellets from the copepod experiments in this study is a result of a concentrating effect during grazing of components which were originally present in the alga in trace abundance. This is supported by the occurrence of purpurin-18-phytyl ester in the algal culture in the diatom experiment.

There was no indication of oxygenated products in the protozoan experiment with *I. galbana* and the presence of a minor amount of chlorophyllone *a* in the green alga may be due to the fact that this sample was not an individual culture but was separated from the total mixture (see Chapter 6) so the high abundance of such products in both of the protozoan experiments and in similar studies (e.g. Strom, 1993) suggest an alternative or additional pathway of transformation is occurring. It seems likely that this difference could arise due to differences in the digestion pathways involved in protozoa which may in fact be leading to activation of endogenous algal enzymes which are responsible for the production of these components with increased hydrolysis activity (see above) leading to the formation of the hydrolysed counterparts of the products observed in the copepod experiments.

7.1.8.6. General significance and potential as grazing indicators

The chl transformation products phaeophytin *a* and phaeophorbide *a* have long been recognised as products of zooplankton herbivory (e.g. Currie, 1962; Shuman and Lorenzen, 1975). More recently these products and their pyro counterparts have been proposed as indicators of macro and mesozooplankton grazing (e.g. Head and Harris, 1992; Otsuki *et al.*, 1993; Strom, 1993). However, interpretation of such components as markers is complicated as they can also derive from senescent algae (e.g. at the end of a phytoplankton bloom). Their production in senescent algal systems has been demonstrated in a number of laboratory studies (e.g. Spooner *et al.*, 1994a, b; Louda *et*

al., 1998). Therefore, finding products which are specific to a single process is of significant interest both to pigment oceanographers as well as geochemists.

To date, the production of SCEs has only been demonstrated directly in grazing experiments (Harradine *et al*., 1996b; and this thesis) or from the guts and faecal pellets of salps which had been grazing *in situ*; therefore, they are believed at present to be directly representative of macro and mesozooplankton grazing. The recently discovered carotenol chlorin esters (CCEs) have been postulated as markers specifically of grazing on diatoms as the carotenols involved were transformation products of fucoxanthin (**XXVI**), a pigment commonly used as a diatom marker, although it can also be found in haptophytes as well as in a number of other algal species. The hopanol chlorin ester found in Lake Valencia (Venezuela; Harradine *et al*., 1996b) bottom sediment would seem to be a specific indicator of grazing on cyanobacteria and the tetrahymanol chlorin ester from the same location indicative of grazing on or by ciliates.

As none of the assigned (non SCE) species observed in this work have been found to originate from a single source it would not be possible to use them as specific indicators of, for example, mesozooplankton grazing on haptophytes. There was a high abundance of dephytylated transformation products (i.e. chlorophyllone *a* and *b*, purpurin-18, 13^2-oxopyrophaeophorbide *a*) excluding pyrophaeophorbide *a* (widely considered to be an indicator of macro and mesozooplankton grazing), produced during microzooplankton herbivory, compared to that during the copepod (mesozooplankton) experiments. This may simply be apparent in this work due to the high cell densities used in the copepod experiments, which result in short gut passage time and low conversion efficiencies (e.g. Downs, 1989). Nevertheless, this phenomenon could potentially be used as an indicator of high grazing pressure from a microzooplankton population if such components are observed, for example, during an algal bloom (e.g. Strom, 1993).

7.2. FUTURE WORK

7.2.1. General

This study has demonstrated production of SCEs when the copepod *Calanus helgolandicus* grazed on certain members of the four major algal divisions (Chlorophyta, Haptophyta, Bacillariophyta and Dinophyta). It has been shown that a variety of factors can lead to changes in the phytoplanktonic sterol distribution prior to esterification and also that the proportion of total chlorins excreted in faecal pellets accounted for by SCEs is variable according to substrate. In order to further establish the applicability of SCEs as indicators of phytoplankton community structure it follows that further studies should be carried out and such studies could be combined with further investigation of the unusual oxygenated products observed in a number of the copepod studies as well as the heterotrophic dinoflagellate experiments. Suggestions for this work follow.

7.2.2. Further copepod studies

- The large scale dinoflagellate experiment (*P. micans*) should be repeated on an even larger scale so that the SCE fractions can be isolated and the SCE sterols obtained by hydrolysis or $LiAlH_4$ reduction and analysed by GC and GC-MS to assess the contributions from the sterols which co-elute as SCEs. This experiment should also involve repeating the ageing and sterilisation study using accurately quantified pellet aliquots to allow quantification of pigment and sterol losses during ageing as well as a longer period of ageing. The ageing study could also involve simulation of pellet sinking through the water column (using a rotating wheel) as pellets are known to degrade faster whilst sinking than when stationary as they are more rapidly colonised by bacteria.

- Repeating the *P. carterae* experiment in order to analyse the pellet free sterols may help explain the absence of two of the algal sterols from the SCE fraction. If these

sterols are absent from the pellets they have clearly been efficiently assimilated by the copepod (for the production of cholesterol).

- Experiments with an algal substrate with a highly complex sterol distribution (e.g. *Scrippsiella trochoidia*; Harvey *et al.*, 1987) to further examine the alteration of substrate sterol distributions prior to esterification.

- Given the variability in production of SCEs from haptophytes a further experiment involving *Emiliania huxleyi*, the most abundant bloom forming haptophyte species in the marine environment, should be performed and quantified (see below) to assess the extent of production of SCEs during grazing on this substrate.

- Further experiments should be performed using members of the less abundant algal divisions such as the Cryptophyta, Euglenophyta, Chrysophyta and Eustigmatophyta to establish SCE production from these algal divisions, as well as to investigate the extent of production of SCEs and degree of alteration of the substrate sterol distribution.

- Further experiments with members of the Chlorophyta should be performed, involving full budgeting of the chl *a* and chl *b* contribution to the SCEs to compare the relative abundance of the pyrophaeophorbide *a*:pyrophaeophorbide *b* pools with the relative abundance of chl *a* and chl *b*, as it has been reported that zooplankton faecal pellets show significant enrichment in chl *b* (Vernet and Lorenzen, 1987b).

- Experiments involving full budgeting of the amount of ingested algal material which is converted to SCEs are needed to assess the efficiency/extent of conversion of algal chl to SCEs. Such studies would also include performing experiments at different dietary concentrations (i.e. simulating oligotrophic, eutrophic and bloom conditions) to assess the amount of SCE produced at high food concentrations where gut passage time is short and the extent of chl transformation (i.e. production of phaeophorbides: phaeophytins) is low, as well as at low concentrations where gut passage time increases and the extent of chl transformation is high. This may also provide insight

into the possible mode of production of SCEs i.e. transesterification of pyrophaeophytin *a* or esterification of pyrophaeophorbide *a*.

- Long term ageing studies with pellets derived from fresh and senescent algal cultures are needed to further investigate the accumulation of oxygenated products. Simulation of sinking should also reveal if such products are "concentrated up" relative to other chlorins including SCEs as they pass through the water column.

- Feeding experiments with algae known to contain a high abundance of long chain alcohols should be performed to attempt to establish the production of long chain alcohol chlorin esters. Synthesis of standards of such components would help facilitate their identification in complex sedimentary chlorin distributions as well as in faecal pellets.

- As no carotenoid chlorin esters (CCEs) were observed during the diatom study further experiments with other diatom species at differing concentrations and possibly with senescent cultures (to date only fucoxanthin alteration products have been observed in such components; Goericke *et al.*, 1999) should be performed to establish their production during herbivorous grazing.

- In order to attempt to demonstrate the production of phaeophorbide *a* steryl esters feeding experiments should be performed with species known to contain a high chlorophyllase content (i.e. diatoms; e.g. *Phaeodactylum tricornutum*). It is possible that rapid hydrolysis of the phytyl ester due to the high chlorophyllase content may result in a competitive pathway resulting in the production of phaeophorbide esters more quickly than the production of the decarbomethoxylated species (i.e. pyrophaeophytin *a*, pyrophaeophorbide *a*).

7.2.3. Other Experiments

- Herbivorous species of marine macrozooplankton (e.g. salps, euphausiids) and other mesozooplankton (e.g. other copepods) should be used in further feeding experiments involving full pigment budgets (see above) to determine the extent of SCE production in non microzooplankton species and to determine if/how herbivore species affects the efficiency of the esterification process.

- Experiments with other microzooplankton species, especially ciliates, should be performed to determine if SCEs can be produced by any types of microzooplankton. Such experiments would also allow further examination of the production of 13^2-hydroxychlorophyllone *a* by microzooplankton.

- The origin of triterpenoid chlorin esters (Harradine *et al.*, 1996a) is not yet known, so experiments involving for example, ciliates feeding on "sterol poor" diets should be performed to determine if the tetrahymanol pyrophaeophorbide *a* ester is produced in this manner. Experiments with larger species e.g. filter feeding salps or smaller species e.g. ciliates grazing directly on cyanobacteria could be performed to investigate the production of the (22*R*)-30α,30β-dihomohopan-30β-ol pyrophaeophorbide *a* ester.

- Grazing experiments involving combinations of different herbivores and substrates from lacustrine environments should be performed to investigate the production of SCEs in other aquatic environments.

- To confirm the findings indicating that alteration of the sterol distributions prior to esterification is a result of utilisation by the copepod to produce cholesterol, tracer experiments involving conventional biochemical techniques (^{13}C or ^{14}C labelling) should be carried out to determine the face of labelled sterols the production of SCEs.

- Further investigation of the relationship between sedimentary SCE and bound sterols (Pearce *et al.*, 1998) should be carried out using samples from a variety of locations

(e.g. marine, lacustrine, coastal, open ocean, oligotrophic, eutrophic, oxic, anoxic) in order to determine if bound sterols are produced at the same time as SCEs or not.

Chapter 8

EXPERIMENTAL

8.1. GENERAL

8.1.1. Glassware

All glassware to be used for chlorin and sterol work was cleaned in a bath of 'Micro' detergent (left to soak overnight), rinsed with tap water and deionised water and then oven dried. Before use, glassware was rinsed with HPLC grade acetone or DCM as appropriate.

8.1.2. Solvents

All solvents used were HPLC grade (Rathburn). Water was double distilled or Milli-Q grade.

8.2. FEEDING EXPERIMENTS

8.2.1. Copepod Experiments

All copepod feeding experiments were carried out in collaboration with Plymouth Marine Laboratory (PML) and were similar to that described for the preliminary study by Harradine *et al.* (1996a).

The algal cultures were grown in F/2 culture medium at 15°C (Guillard, 1975). Grazing took place in multiple 5 L glass beakers (Table 8-1). The algal cell densities are shown in Table 8.1 and a second aliquot of the culture at the same equivalent concentration was added to each of the feeding beakers after 24 h. The cell concentrations were estimated by multisizer counts on the main culture. Culture samples (usually 250 ml of stock culture) for pigment and sterol analysis were filtered onto GF/F filters (0.6 μm) and frozen immediately at -20°C until analysis.

Expt.	Alga	PCC[a] No.	No. beakers	No. control beakers	Cell density (cells ml^{-1})	No. animals per beaker	Ageing
1a	*Thalassiosira weissflogii* (Diatom)	541	6	2	1.3 E^4	120	Pellets from 2 beakers aged 30 d
1b	*Thalassiosira weissflogii* (Diatom)	541	16		1.3 E^4	95	*see section 8.2.1.1*
2a	*Prorocentrum micans* (dinoflagellate)	97a	6	2	7.2 E^2	90	*see section 8.2.1.2*
2b	*Prorocentrum micans* (dinoflagellate)	97a	16	2	7.0 E^2	100	*see section 8.2.1.3*
3	*Alexandrium tamarensis* (dinoflagellate)	173a	6	1	6.4 E^2	90	Pellets from 2 beakers aged 30 d
4	*Dunaliella tertiolecta* (Chlorophyte)	83	2	0	1.0 E^4	80	No ageing
5	*Chlamydomonas reginae* (Chlorophyte)	399	2	0	1.0 E^4	80	No ageing
6a	*Tetraselmis suecica* (Prasinophyte)	305	2	0	1.0 E^4	80	No ageing
6b	*Tetraselmis suecica* (Prasinophyte)	305	10	2	1.0 E^4	100	No ageing
7	*Isochrysis galbana* (Haptophyte)	I	2	1	6.0 E^4	90	No ageing
8	*Coccolithus pelagicus* (Haptophyte)	182q	6	1	6.0 E^3	90	500 pellet aliquots aged but chlorin content too low.
9	*Crycosphaera carterae* or *Pleurochrysis carterae* (Haptophyte)	17	8	1	7.7 E^4	100	No ageing

[a] PCC - Plymouth Culture Collection

Table 8-1. *Copepod feeding experiments.*

Females and stage V of the copepod *Calanus helgolandicus* were collected from the Plymouth coastal area (site L4; figure 8.1) and were starved for 24 h in filtered sea-water (0.6 μm) prior to the experiment. A sample (typically 50-100) of the starved animals were collected for sterol analysis and 80-120 (Table 8-1) animals were added to each feeding beaker and allowed to graze for 48 h. Where sufficient culture was available, controls containing only the algal culture at the feeding experiment cell densities were run alongside the feeding experiments to confirm that SCEs were not produced by the culture alone.

After 48 h the animals were removed by filtering through a 500 μm mesh and retained for sterol analysis. The faecal pellets were then separated from any remaining algae by filtration through a 35 μm mesh and collected by filtration on to GF/F filters or prepared for ageing experiments (Table 8-1).

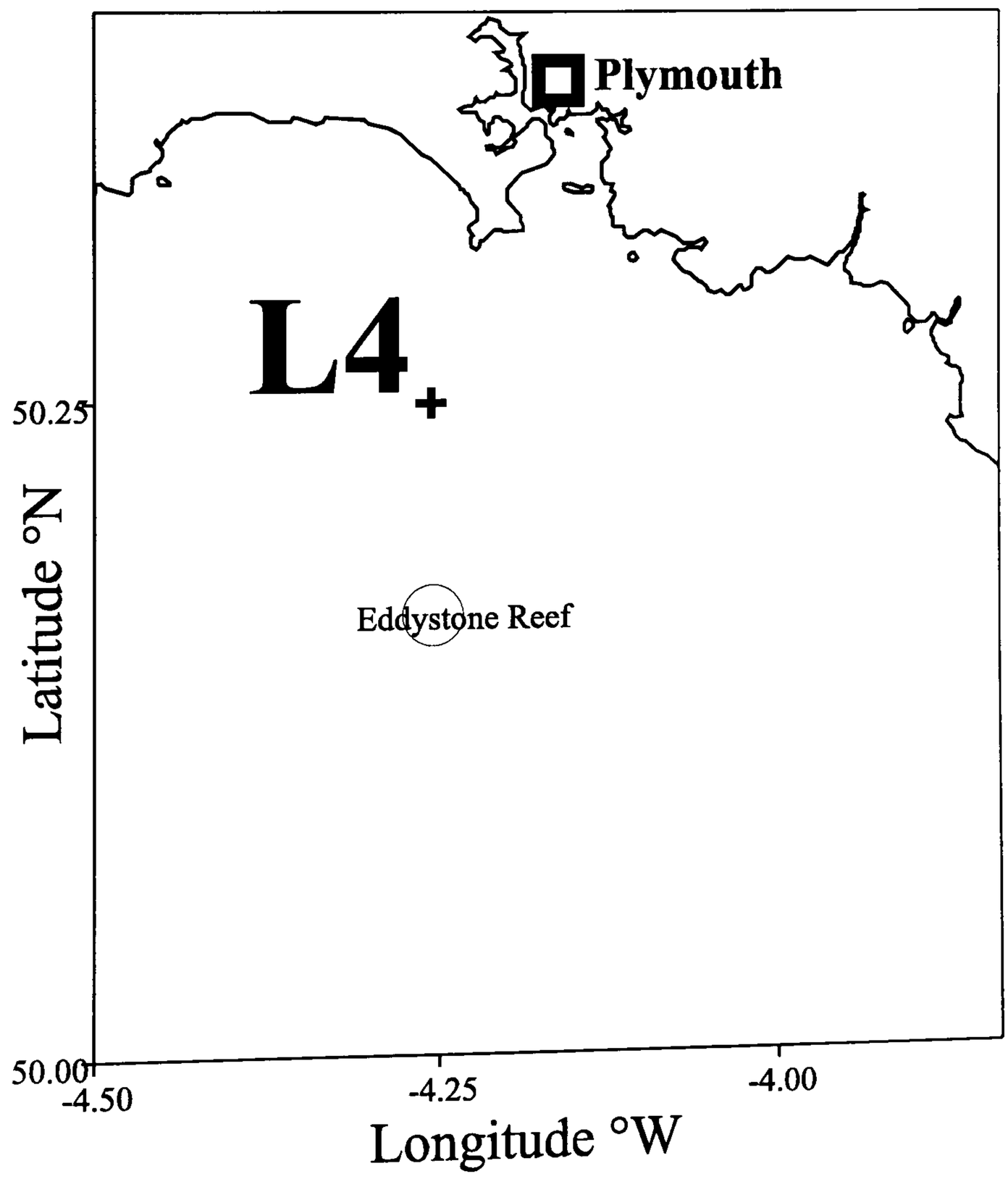


Figure 8-1. *Map of location of site L4 (English Channel, UK)*

8.2.1.1. Pellet Ageing (large scale T. weiss experiment 1b)

Faecal pellets from 4 of the feeding beakers were combined and 12 aliquots of 500 pellets were isolated manually by pipette during microscopic examination of the pellets. Four of the aliquots were filtered immediately and frozen; the remaining 8 aliquots were aged in the dark in filtered seawater at 15°C in covered 500ml glass beakers for a period of 10 or 30 d before filtering. Before extraction, pellet aliquots were spiked with a synthetic C_{32} hopanol chlorin ester (Harradine *et al.* 1996a) as internal standard and extracted in the usual way (see below). Pellets from the remaining 12 beakers were collected in groups of 4 beakers. Pellets from one group were filtered immediately, the other groups being aged for 10 and 30 d respectively under the same conditions as the 500 pellet aliquots.

8.2.1.2. Pellet Ageing (normal scale P. micans experiment 2a)

The pellets from 3 of the feeding beakers were separated from any remaining algae (35 μm mesh) and filtered immediately. The pellets from the remaining 3 beakers were combined and 4 aliquots of 100 pellets were separated manually (see above). The aliquots were aged for 0, 2, 7, and 31 d respectively, in the dark in filtered seawater at 15°C. Preliminary analysis of the sample aged for 2 d revealed that pigment concentrations were very low, so the samples aged for 7 and 31 days were combined before extraction and analysis.

8.2.1.3. Pellet Ageing (large scale P. micans experiment 2b)

Faecal pellets from all 16 feeding beakers were collected and made up to a volume of 3 L in filtered seawater (0.6 μm). Three aliquots (50ml) were taken for microscopic counting to determine the average number of pellets in each aliquot. A further 14 aliquots (200ml) of suspended pellets were taken for ageing experiments. Four were filtered immediately, four were aged (as above) for 8 d, four were aged for 29 d and 2 were sterilised with $HgCl_2$ and also aged for 29 d. Before extraction, pellet samples were spiked with a synthetic C_{32} hopanol chlorin ester (Harradine *et al.* 1996a) and pregnen-3β-ol as internal standards then extracted in the usual way (see below).

8.2.2. *Oxyrrhis Marina* Feeding Experiments

The heterotrophic dinoflagellate *Oxyrrhis marina* was grown up in F/2 culture medium at 15°C (Guillard, 1975). The culture also contained an unknown green alga on which the protozoan feeds during growth. Individual cells of the protozoan were isolated from this mixture and transferred to a culture of *Isochrysis galbana* also grown up in F/2 culture medium. Cultures were divided to promote further growth and either diluted with more F/2 culture medium (*O. marina/green alga*) or by the addition of fresh *I. galbana* culture *ca*. 1-2 times weekly.

When the *O. marina* and green alga culture had been "bulked" to *ca*. 7.5 L a 2.5 L aliquot was separated yielding fractions containing mainly green alga or *O. marina*. The remaining 5 L of bulk culture was harvested by filtering directly on to GF/F filters to yield total particulates (as *O. marina* is a protozoan it does not produce discreet faecal pellets so samples of total particulates were collected).

Eight days prior to harvesting, the *O. marina / I. galbana* culture was divided into two aliquots. Two days prior to harvesting the first aliquot was fed with fresh *I. galbana* culture (total culture volume = 5 L). The second aliquot was not fed again and a high proportion of the *I. galbana* cells had been cleared due to grazing at the point of harvesting (total culture volume = 4 L). A sample of fresh *I. galbana* was also taken for comparison of pigment distributions.

8.2.3. Microzooplankton (500-200μm) and Cyanobacterium Feeding Experiment

A zooplankton were collected from Site L4 (Figure 8.1) from a depth of *ca*. 10 m. The microzooplankton fraction (200-500 μm) was separated by filtering through a 500 μm mesh followed by collection on a 200 μm mesh. The animals were starved overnight in filtered seawater (0.6 μm) to allow the animals to empty their guts. Two batch cultures of the marine cyanobacterium *Synechococcus* sp. (Strain DC_2) were grown up under routine conditions (Wilson, personal communication). One culture was added to a 20 L beaker and diluted with an equal volume of filtered seawater (0.6 μm) as the cyanobacterium was grown up at 25% salinity. The second culture was divided into two

and half was refrigerated overnight (4°C; immediately prior to commencement of grazing to induce senescence) and the other half was frozen overnight (*ca.* -20°C; immediately prior to commencement of grazing to induce senescence). The two senescent cultures were separately added to 10 L beakers respectively and diluted with filtered seawater (as above). Equal volumes (500 ml) of animals were added to each of the three beakers containing the diluted cultures. Aliquots of each culture were taken before dilution for pigment analysis. An aliquot (500 ml) of the total mixture from each beaker was taken after 24 h and 48 h of grazing and the total bulk remaining contents were also filtered after 48 h.

8.3. EXTRACTION OF SAMPLES

All algal, pellet and animal samples on GF/F filters were divided by cutting the filters into smaller pieces then extracted by successive sonication in acetone (0°C, 15 min) and centrifugation (10 min at 3000 rpm), until the supernatant was colourless (typically x 5). The extract was transferred in acetone to a vial and the solvent removed under a stream of O_2-free nitrogen.

8.4. PIGMENT ANALYSIS

8.4.1. High Performance Liquid Chromatography

Reversed-phase (RP) HPLC (Waters MS600 silk quaternary) was carried out using a Phenomenex ODS3 column (150 mm x 4.6 mm i.d., 1 ml min^{-1}) with an ODS3 guard column (30 x 4.6 mm i.d.). The use of an on-line Waters 990, 991 or 996 photodiode array (PDA) detector provided electronic spectra of individual components from 350 to 700 nm. The same gradient elution programme (Table 8.2) was used in most instances except for the large scale *T. suecica* experiment (Expt 6b, Table 8.1) where the programme was modified slightly to facilitate separation of pyrophaeophytin *b* from phaeophytin *a* (Table 8.3).

Time (min)	% Acetone	% H_2O	% MeOH
0	0	20	80
5	0	20	80
15	30	10	60
25	30	10	60
35	60	10	30
45	90	5	5
95	90	5	5
100	0	20	80

Table 8-2. *Typical gradient elution programme for HPLC.*

Time (min)	% Acetone	% H_2O	% MeOH
0	0	20	80
5	0	20	80
15	30	10	60
25	30	10	60
35	60	10	30
45	80	10	10
55	90	5	5
95	90	5	5
100	0	20	80

Table 8-3. *Gradient elution programme for HPLC of pigment samples from T. suecica experiment (6b).*

8.4.2. High Performance Liquid Chromatography - Mass Spectrometry

The HPLC system (as above, or with a Waters 996 PDA with Millenium[32] software) was linked to a Finnigan MAT TSQ 700 quadrupole mass spectrometer *via* a Finnigan MAT atmospheric pressure chemical ionisation (APCI) interface (Harris *et al.*, 1995a). Interface conditions were typically: vaporiser 450°C; capillary 300°C; corona 7μA (≈ 5 kV), sheath gas pressure 50 psi with the auxillary gas turned off.

8.5. STEROL ANALYSIS

8.5.1. Free Sterol Isolation

A fraction containing the free alcohols was obtained from both the feeding experiment culture and animal samples by TLC of the total extract on silica, by eluting with DCM and using sitosterol ($C_{29}\,\Delta^5$ sterol), friedelin and lupeol as standards.

8.5.4. Derivatisation

Free sterol fractions were derivatised by addition of a few drops of bis(trimethylsilyl) acetamide and heating (60°C, 4 h).

8.5.5. Gas Chromatography

GC analyses were carried out using either a Carlo Erba HRGC 5300 Mega series gas chromatograph or a Hewlett Packard 5890 Series II gas chromatograph (both equipped with an on column injector) fitted with a 50 m x 0.32 mm id CP Sil-5CB column. Data were collected using Minichrom or Xchrom (Carlo Erba) or HP Chem Station (Hewlett Packard). Alcohols were analysed as their trimethylsilyl derivatives (see above) and injected (1.0 μl) in DCM solution. The temperature programme used was typically [40-200°C (10°C min^{-1}, hold 5 min.) -300°C (3°Cmin^{-1}, hold 30 min.)]. The carrier gas was hydrogen.

8.5.6. Gas Chromatography - Mass Spectrometry

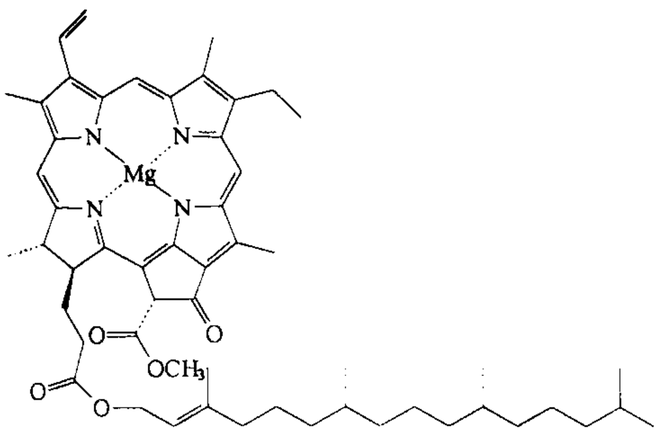
GC-MS analysis was performed using a Carlo Erba HRGC 5160 Mega series chromatograph linked to a Finnigan MAT 4500 quadrupole mass spectrometer operated under similar conditions as above, but employing helium as the carrier gas. The spectrometer was operated in the EI mode (ionising energy 70 eV; source temperature 170°C; filament current 250 μA; flow rate 40 cm s^{-1}; scan time [m/z 50-650] of 1 s).

Data were collected using an INCOS 2300 system and components were assigned by comparison of mass spectra and relative retention times with literature data.

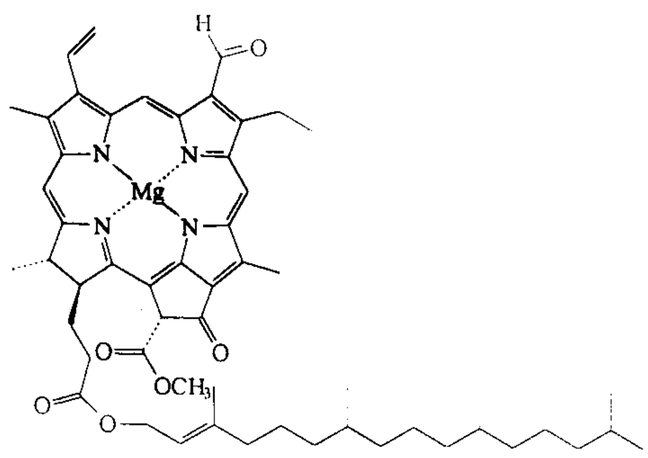
STRUCTURES

STRUCTURES

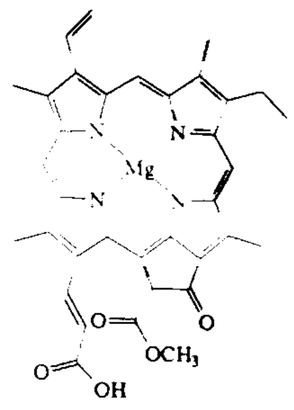
I



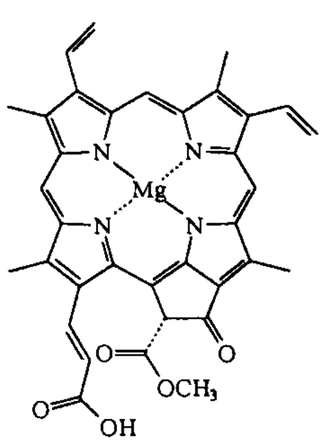
II



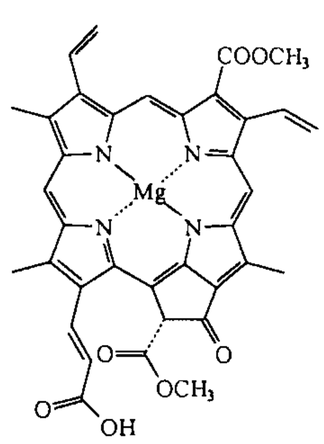
III



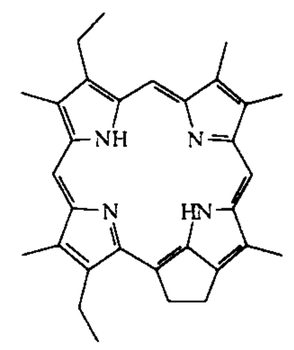
IV



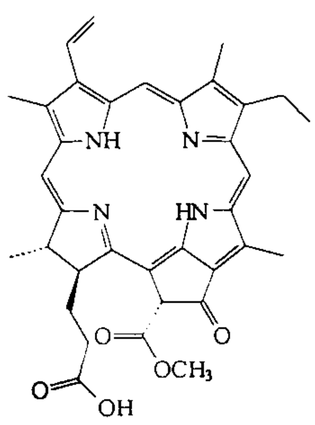
V



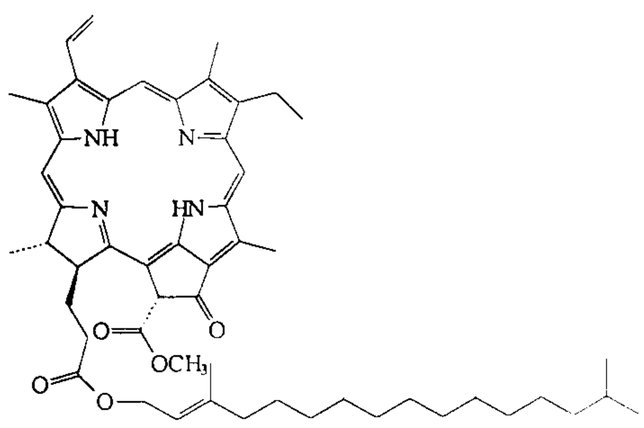
VI



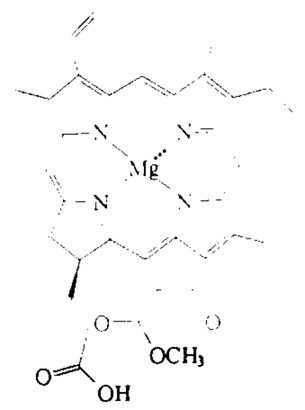
VII



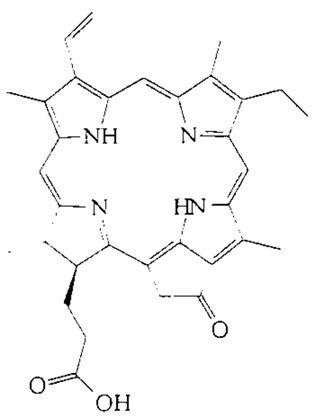
VIII



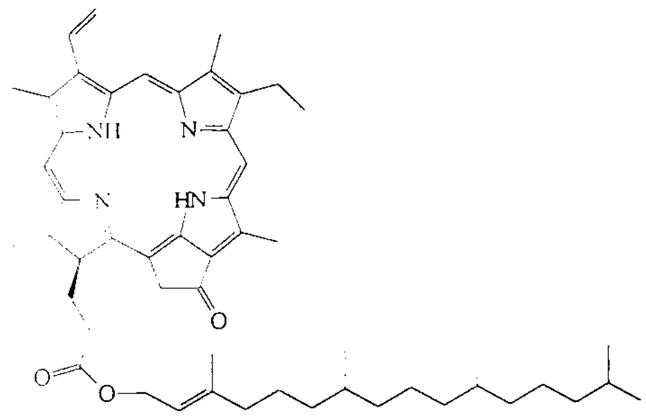
IX



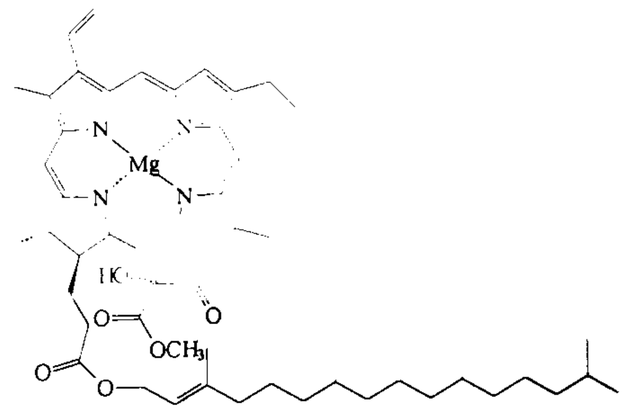
X



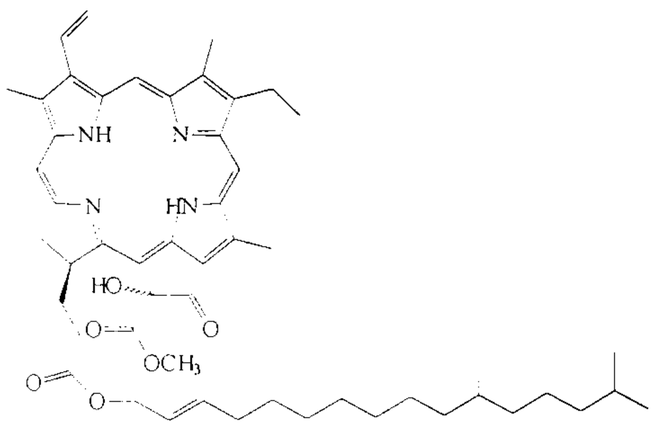
XI



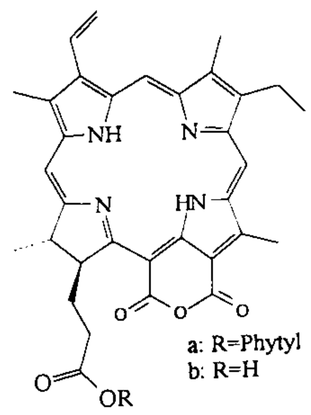
XII



XIII

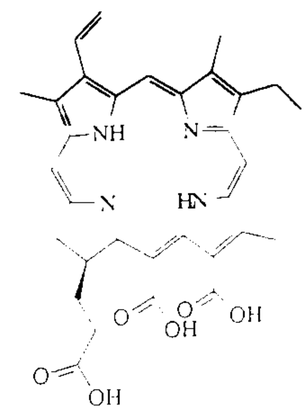


XIV

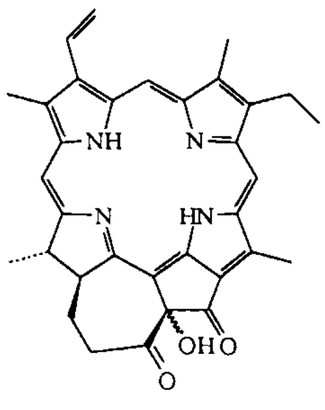


a: R=Phytyl
b: R=H

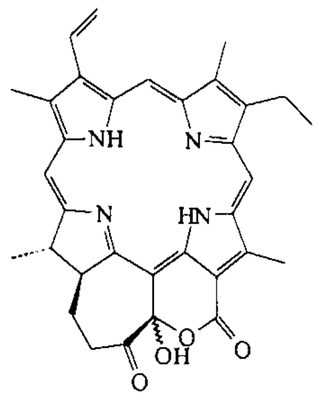
XV



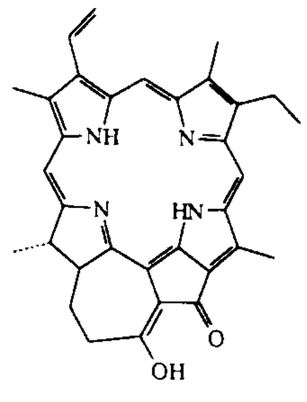
XVI



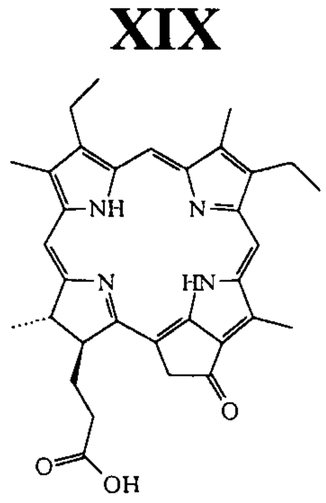
XVII



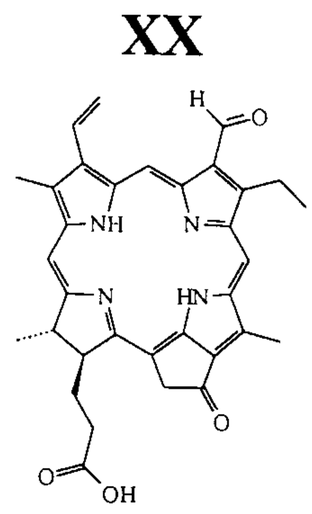
XVIII



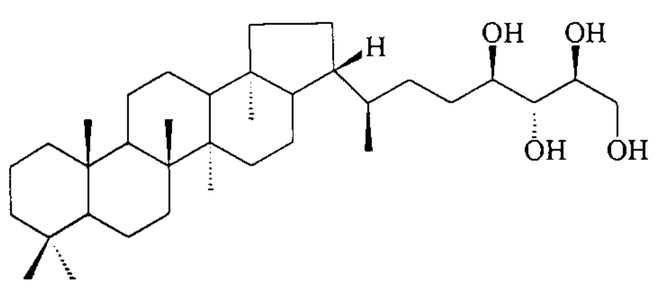
XIX



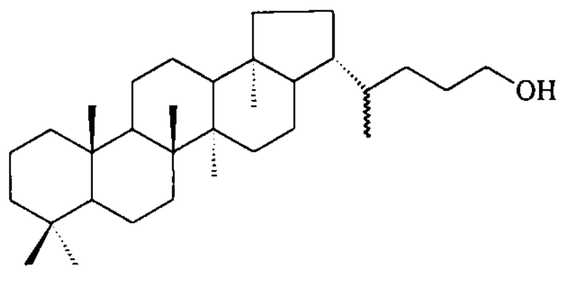
XX



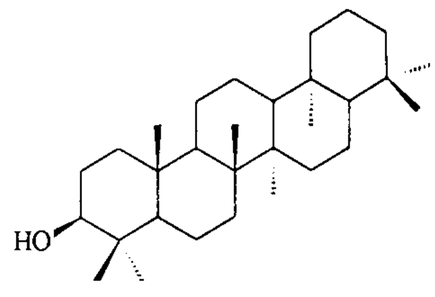
XXI



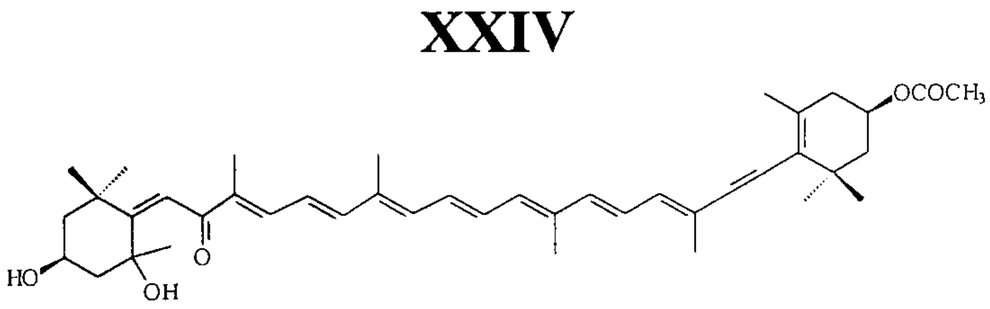
XXII



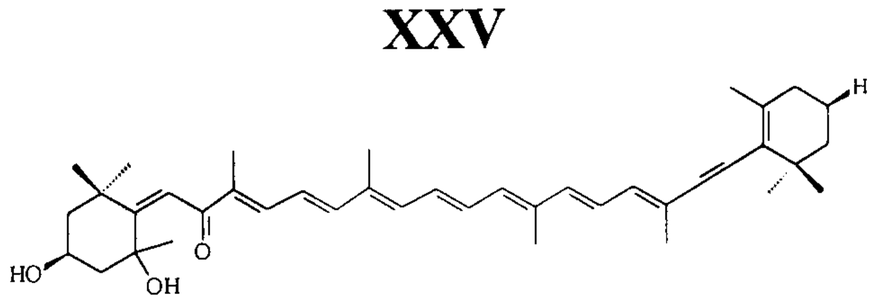
XXIII



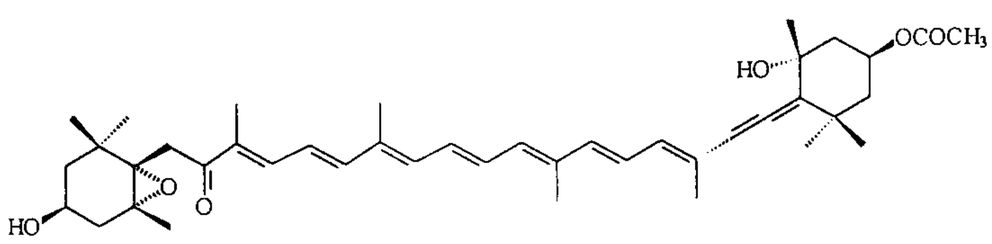
XXIV



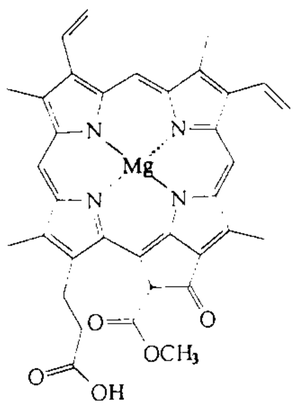
XXV



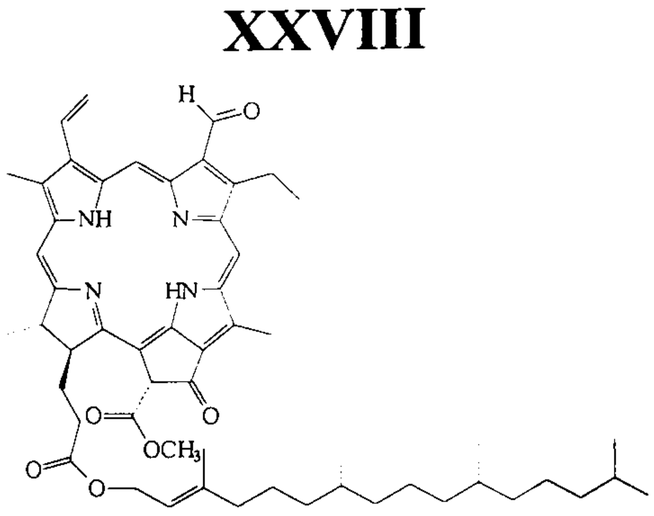
XXVI



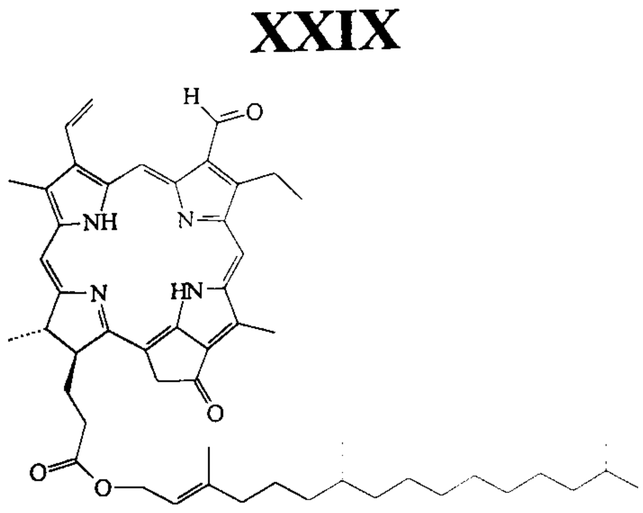
XXVII



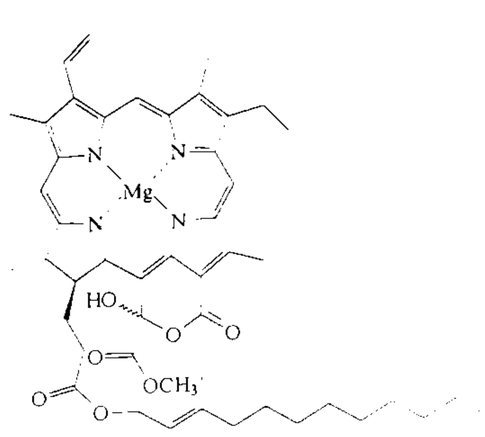
XXVIII



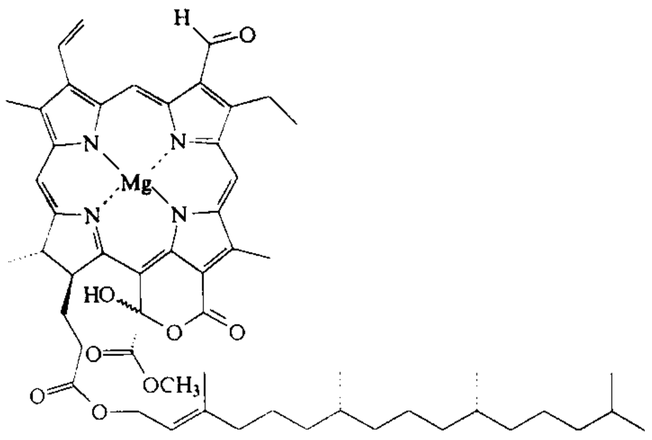
XXIX



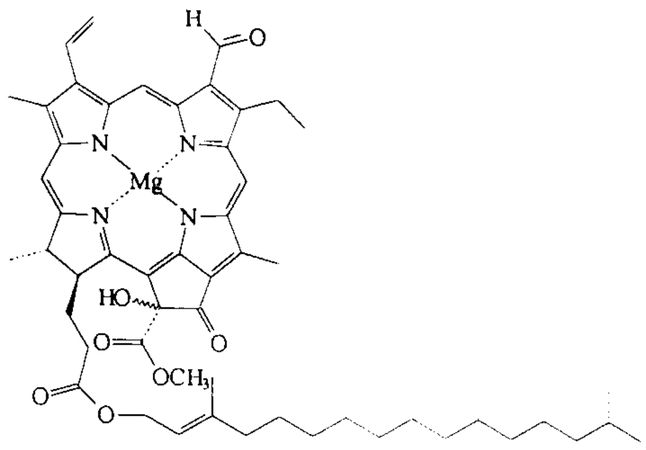
XXX



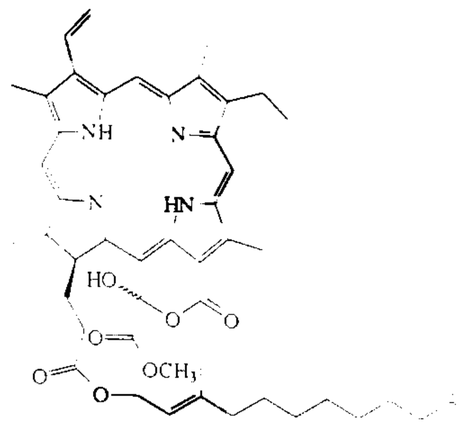
XXXI



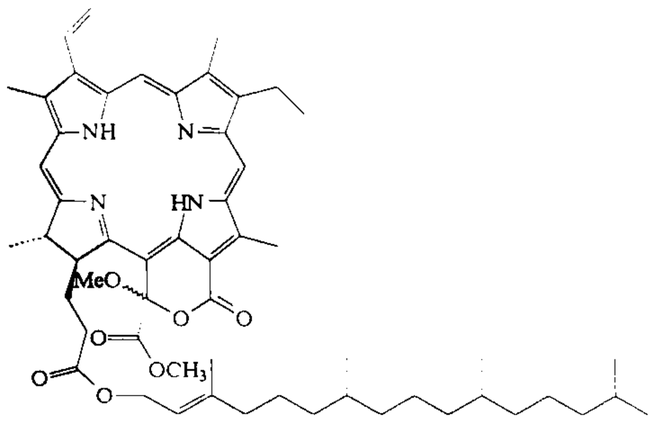
XXXII



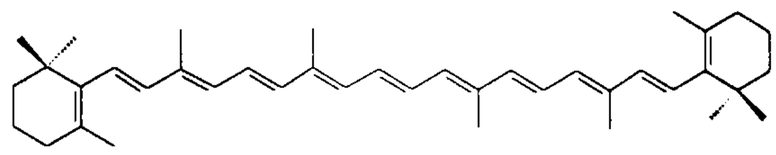
XXXIII



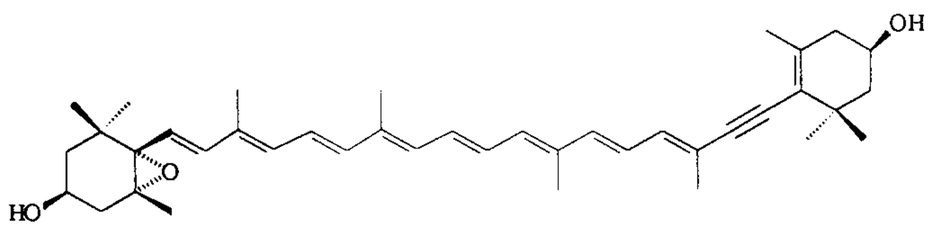
XXXIV



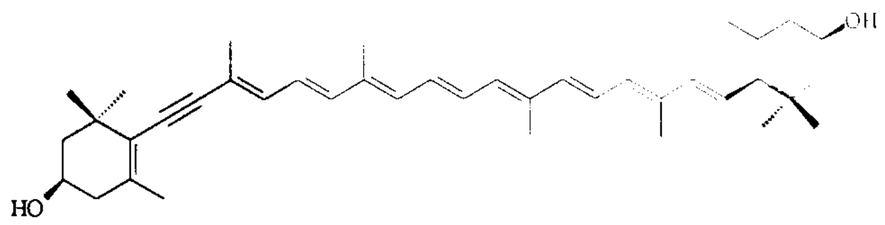
XXXV



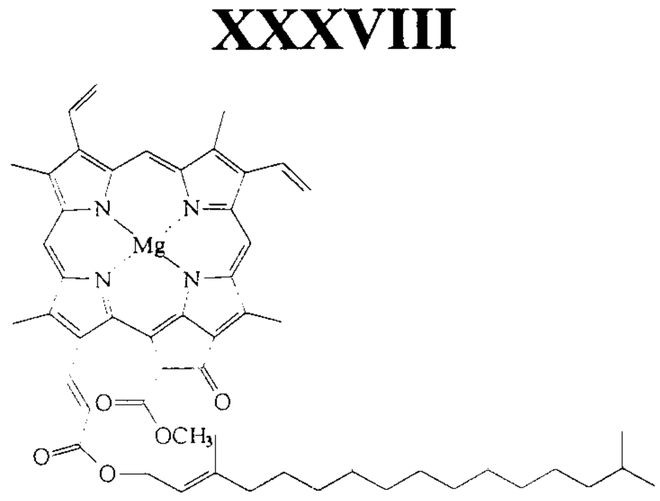
XXXVI



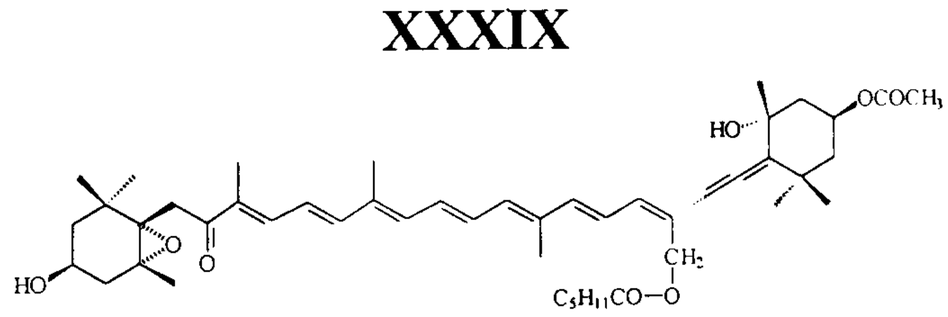
XXXVII



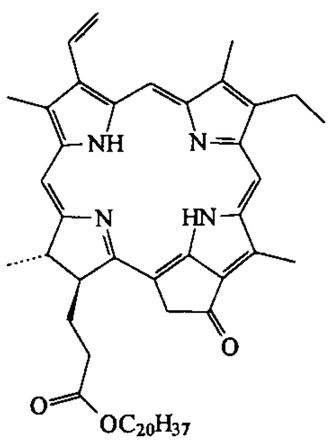
XXXVIII



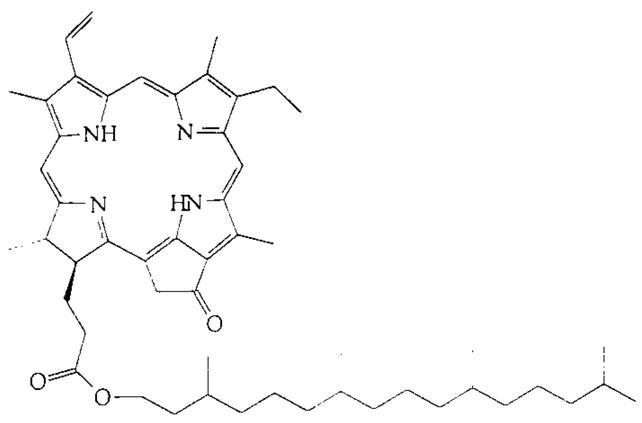
XXXIX



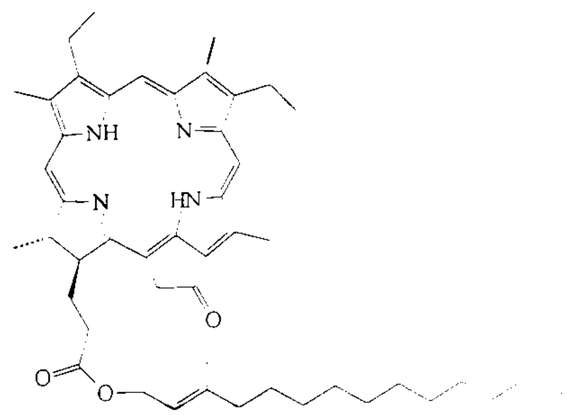
XXXX



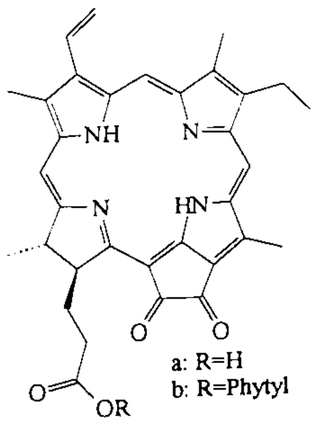
XXXXI



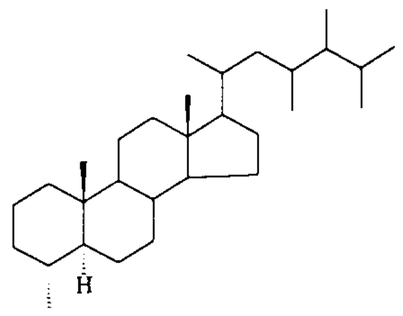
XXXXII



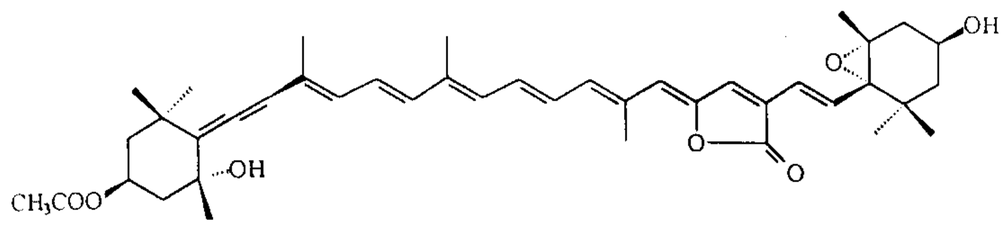
XXXXIII



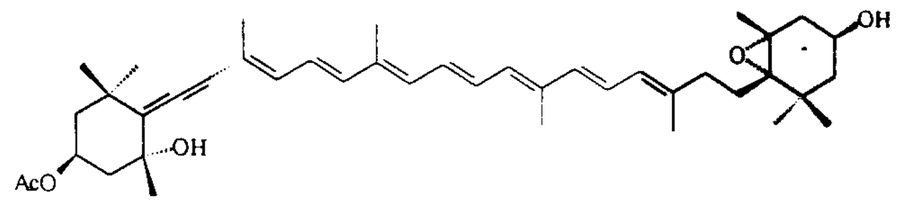
XXXXIV



XXXXV



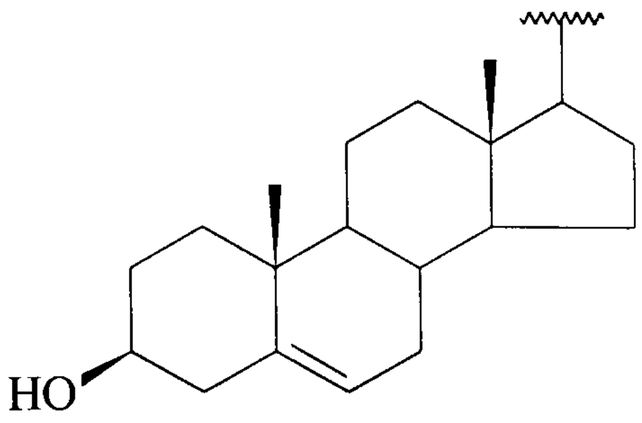
XXXXVI



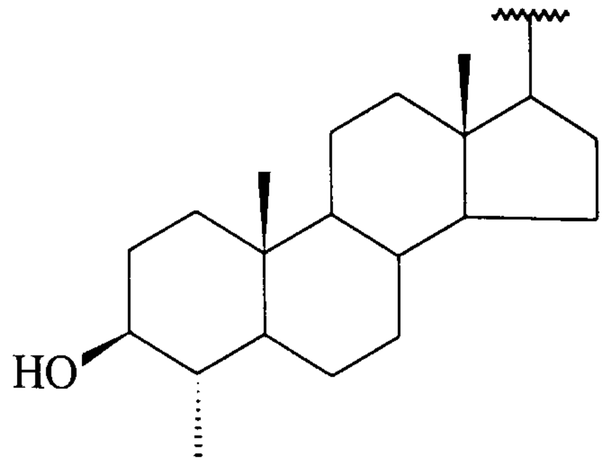
XXXXVII

XXXXIX

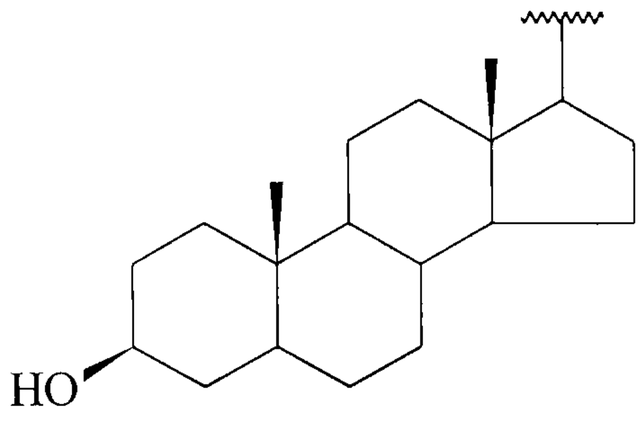
STEROL STRUCURES



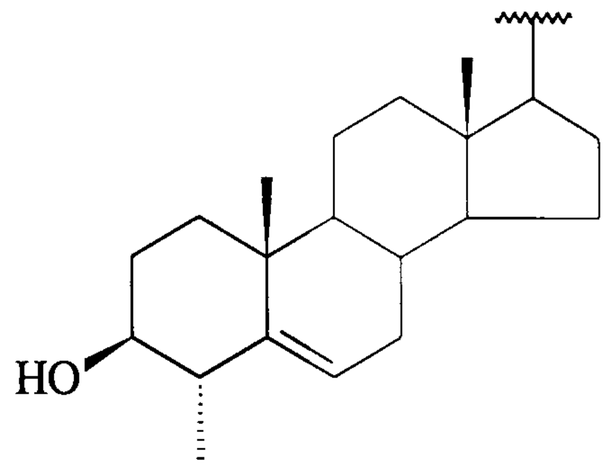
A



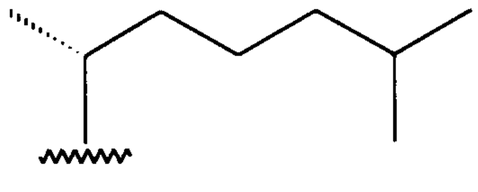
B



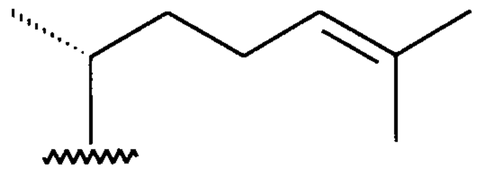
C



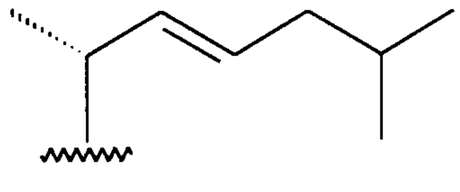
D



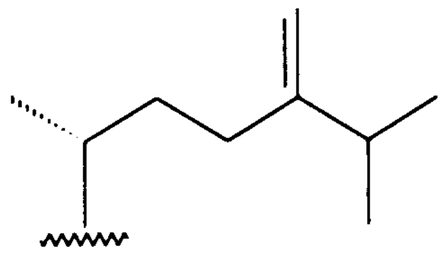
1



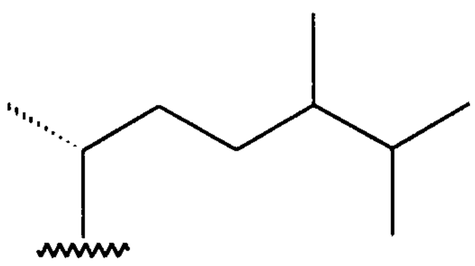
2



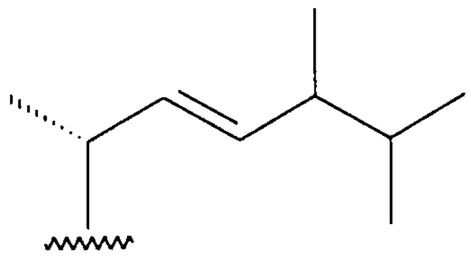
3



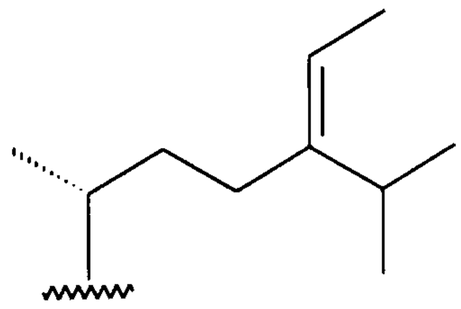
4



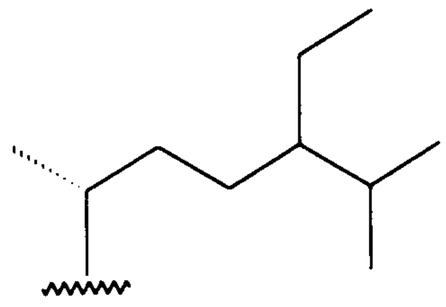
5



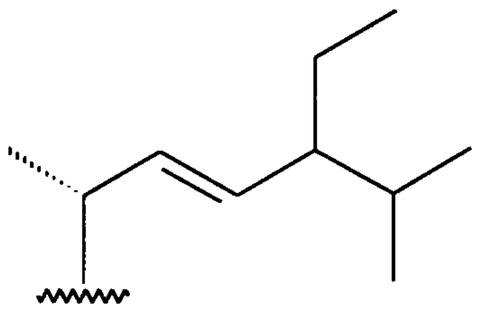
6



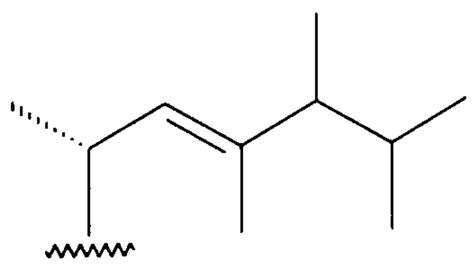
7



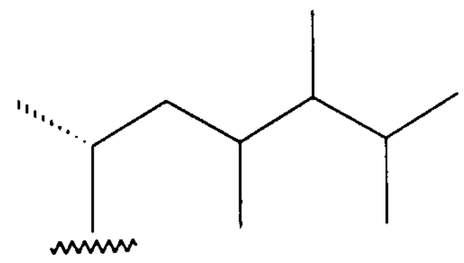
8



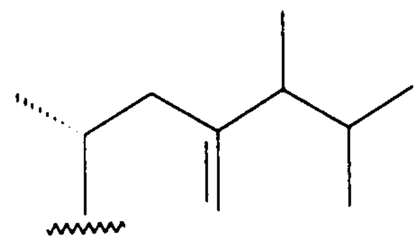
9



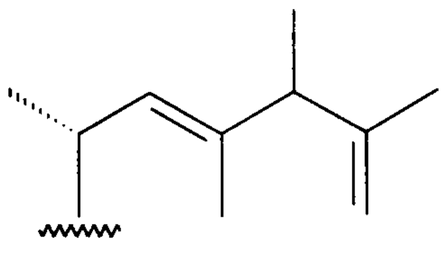
10



11



12



13

REFERENCES

REFERENCES

Aiken, J., Moore, G.F. and Holligan, P.M. (1992) Remote sensing of oceanic biology in relation to global climate change. *Journal of Phycology* **28**, 579-590.

Baker, E.W. and Louda, J.W. (1986) Porphyrins in the geological record. In: *Biological Markers in the Sedimentary Record*, ed. R.B. Johns pp. 125-225. Elsever, Amsterdam.

Barlow, R.G., Burkill, P.H. and Mantoura, R.F.C. (1988) Grazing and degradation of algal pigments by marine protozoan *Oxyrrhis marina*. *Journal of Experimental Marine Biology and Ecology* **119**, 119-129.

Barlow, R.G., Mantoura, R.F.C., Gough, M.A. and Fileman, T.W. (1993) Pigments signatures of the phytoplankton composition in the north eastern Atlantic during the 1990 spring bloom. *Deep-Sea Research Part II-Topical studies in Oceanography*. **40**, 459-477.

Barrett, J. and Jeffrey, S.W. (1964) Chlorophyllase and formation of an atypical chlorophyllide in marine algae. *Plant Physiology* **39**, 44-47.

Barrett, S.M., Volkman, J.K., and Dunstan, G.A. (1995) Sterols of 14 species of marine diatoms (Bacillariophyta). *Journal of Phycology* **31**, 360-369.

Bienfang, P.K. (1980) Herbivore diet affects fecal pellet settling. *Canadian Journal of Fisheries and Aquatic Science* **37**, 1352-1357.

Bradshaw, S.A., O'Hara, S.C.M., Corner, E.D.S. and Eglinton, G. (1989) Assimilation of dietary sterols and faecal contributions of lipids by the marine invertebrates *Neomysis integer*, *Scrobiculara plana* and *Nereis diversicolor*. *Journal of the Marine Biological Association of the United Kingdom* **69**, 891-911.

Brown, S.B., Houghton, J.D. and Hendry, G.A.F. (1991) Chlorophyll Breakdown. In: *The Chlorophylls*, ed. H. Scheer, pp.465-489. CRC Press, Boca Ranton, USA.

Buck, K.R. and Newton, J. (1995) Faecal pellet flux in Dabob Bay during a diatom bloom: Contribution of microzooplankton. *Limnology and Oceanography* **40**, 306-315.

Burkill, P.H., Mantoura, R.F.C., Llewellyn, C.A. and Owens, N.J.P. (1987) Microzooplankton grazing and selectivity in coastal waters. *Marine Biology* **93**, 581-590.

Butler, M. and Dam, H.G. (1994) Production rates and characteristics of fecal pellets of the copepod *Acartia tonsa* under simulated phytoplankton bloom conditions: implications for vertical fluxes. *Marine Ecology Progress Series* **114**, 81-91.

Callot, H.J. (1991) Geochemistry of chlorophylls. In: *The Chlorophylls*, ed. H. Scheer H, pp.339-364. CRC Press, Boca Ranton, USA.

Cariou-Le Gall, V., Rosell-Mele, A. and Maxwell, J.R. (1998) *Data Report:* Characterization of distributions of photosynthetic pigments in sapropels from holes 966D and 969C[1]. *Proceedings of the Ocean Drilling Program, Scientific Results* **160**, 297-302.

Caron, D.A., Madin, L.P. and Cole, J.J. (1989) Composition and degradation of Salp fecal pellets: Implications for vertical flux in oceanic environments. *Journal of Marine Research* **47**, 829-850.

Chillier, X.F.D., Gülaçar, F.O. and Buchs, A. (1993) A novel sedimentary lacustrine chlorin: characterisation and geochemical significance. *Chemosphere* **27**, 2103-2110.

Chillier, X.F.D. and Gülaçar, F.O. (1995) Characterisation of chlorin steryl esters in sediments by desorption mass-spectrometry and geochemical signification. *Archives des Sciences* **48**, 29-40.

Conover, R.J., Durvasula, R., Roy, S. and Wang, R. (1986) Probable loss of chlorophyll-derived pigments during passage through the gut of zooplankton and some of the consequences. *Limnology and Oceanography* **31**, 878-886.

Conte, M.H., Volkman, J.K. and Eglinton, G. (1994) 19. Lipid biomarkers of the Haptophyta. In. *The Haptophyte Algae. Systematics Association Special Volume No. 51*, eds. J.C. Green and B.S.C. Leadbeater, pp 351-377. Clarendon Press, Oxford. The Systematics Association, 1994.

Corner, E.D.S., O'Hara, S.C.M., Neal, A.C. and Eglinton, G. (1986) Copepod faecal pellets and the vertical flux of biolipids. In *The Biological chemistry of Marine Copepods*, eds. E.D.S. Corner and S.C.M. O'Hara, pp. 261-321. Oxford Scientific, Oxford.

Cranwell, P.A. and Volkman, J.K. (1981) alkyl and steryl esters in a recent lacustrine sediment. *Chemical Geology* **32,** 29-43.

Currie, R.I. (1962) Pigments in zooplankton faeces. *Nature* **193**, 956-957.

Dagg, M.J. and Walser, W.E. (1987). Ingestion, gut passage, and egestion by the copepod *Neocalanus plumchrus* in the laboratory and in the subarctic Pacific Ocean. *Limnology and Oceanography* **32**, 178-188.

Daley, R.J. (1973) Experimental characterization of lacustrine chlorophyll diagenesis. 2. Bacterial, viral and herbivore effects. *Arch. Hydrobiol.* **72**, 409-439.

Downs, J. (1989) Implications of the phaeopigment, carbon and nitrogen content of sinking particles for the origin of export production. Ph.D. Thesis. University of Washington.

Downs, J.N. and Lorenzen, C.J. (1985) Carbon:pheopigment ratios of zooplankton fecal pellets as an index of herbivorous feeding. *Limnology and Oceanography* **30**, 1024-1036.

Dunbar, R.B. and Berger, W.H. (1981) Fecal pellet flux to modern bottom sediment of Santa Barbara Basin (California) based on sediment trapping. *Geological Society of America Bulletin* **92**, 212-218.

Eckardt, C.B., Carter, J.F. and Maxwell, J.R. (1990) Combined liquid chromatography/mass spectrometry of tetrapyrroles of sedimentary significance. *Energy and Fuels* **4**, 741-747.

Eckardt, C.B., Keely, B.J. Waring, J.R., Chicarelli, M.I. and Maxwell, J.R. (1991a). Preservation of chlorophyll-derived pigments in sedimentary organic matter. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences* **333**, 339-348.

Eckardt, C.B., Keely, B.J. and Maxwell, J.R. (1991b) Identification of chlorophyll transformation products in a lake sediment by combined liquid chromatography-mass spectrometry. *Journal of Chromatography* **557**, 271-288.

Eckardt, C.B., Pearce, G.E.S., Keely, B.J., Kowalewska, G., Jaffé, R. and Maxwell, J.R. (1992) A widespread and abundant chlorophyll transformation pathway in the aquatic environment. *Organic Geochemistry* **19**, 217-277.

Gagosian, R.B. and Heinzer, F. (1979) Stenols and stanols in the oxic and anoxic waters of the Black Sea. *Geochimica et Cosmochimica Acta* **43**, 471-486.

Gagosian, R.B., Smith, S.O., Lee C.L., Farrington, J.W. and Frew, N.E. (1980) Steroid transformations in recent marine sediments. In *Advances in Organic Geochemistry 1979*, eds. A.G. Douglas and J.R. Maxwell, pp. 407-419.

Gagosian, R.B., Volkman, J.K. and Nigrelli, G.E. (1981) The use of sediment traps to determine sterol sources in coastal sediments off Peru. In *Advances in Organic Geochemistry* (ed. M. Bjorøy *et al.*), pp. 369-379. Wiley.

Gauld, D.T. (1957) A peritrophic membrane in calanoid copepods. *Nature, London* **179**, 325-326.

Goad, L.J. (1978) The sterols of Marine invertebrates: composition, biosynthesis and metabolites. In *Marine Natural Products: chemical and biological perspectives*, ed. P.J. Scheuer, Vol.2, pp. 75-172. Academic Press, New York.

Goad, L.J. (1981) Sterol biosynthesis and metabolism in marine invertebrates. *Pure and Applied Chemistry* **51**, 837-852.

Goad, L.J. (1991) Chapter 11. Phytosterols. *Methods in Plant Biochemistry* **7**, 369-433.

Goericke, R., Shankle, A. and Repeta, D.J. (1999) Novel Chlorin Carotenol Esters in Coastal marine Sediments. *Geochimica et Cosmochimica Acta*. In Press.

Goldman, J.C. and Caron, D.A. (1985) Experimental studies on an omnivorous microflagellate: implications for grazing and nutrient regeneration in the marine microbial food chain. *Deep-Sea Research Part A - Oceanographic Research Papers* ***32***, 889-915.

Goldman, J.C., Dennett, M.R. and Gordin, H. (1989) Dynamics of herbivorous grazing by the heterotrophic dinoflagellate *Oxyrrhis marina*. *Journal of Plankton Research* **11**, 391-407.

Gowing, M.M. and Silver, M.W. (1985) Minipellets: a new and abundant size class of marine fecal pellets. *Journal of Marine Research* **43**, 395-418.

Grice, K., Gibbison, R., Atkinson, J.E., Schwark, L., Eckardt, C.B. and Maxwell, J.R. (1996) 1*H*-pyrrole-2,5-diones (maleimides) as indicators of anoxygenic photosynthesis in ancient water columns. *Geochimica et Cosmochimica Acta* **60**, 3913-3924.

Grice, K., Klein Breteler, W.C.M., Schouten, S., Grossi, V. de Leeuw, J.W. and Sinninghe Damsté, J.S. (1998) Effects of zooplankton herbivory on biomarker proxy records. *Paleoceanography* **13**, 686-693.

Guillard R.R.L. (1975) Culture of phytoplankton for feeding marine invertebrates. In : *Culture of marine invertebrate animals.* (eds. Smith W.L and Chaney M.H.), pp. 29-60. Plenum Publishing corp., New York.

Hallegraeff, G.M. (1981) Seasonal study of phytoplankton pigments and species at a coastal station of Sydney: Importance of diatoms and the nanoplankton. *Marine Biology* **61**, 107-118.

Hansen, F.C., Witte, H.J. and Passarge, J. (1996) Grazing in the heterotrophic dinoflagellate *Oxyrrhis marina*: size selectivity and preference for calcified *Emiliania huxleyi* cells. *Aquatic Microbial Ecology* **10**, 307-313.

Harradine, P.J. (1996) Formation, distribution, synthesis and characterisation of naturally occurring chlorophyll transformation products. Ph.D. Thesis. University of Bristol.

Harradine, P.J., Peakman, T.M. and Maxwell, J.R. (1996a) Triterpenoid chlorin esters - water column transformation products of chlorophyll *a*. *Tetrahedron* **52**, 13427-13440

Harradine, P.J., Harris, P.G., Head, R.N., Harris, R.P. and Maxwell, J.R. (1996b) Steryl chlorin esters are formed by zooplankton herbivory. *Geochimica et Cosmochimica Acta* **60,** 2265-2270.

Harradine, P.J. and Maxwell, J.R. (1998) Pyrophaeoporphyrins c_1 and c_2: grazing products of chlorophyll *c* in aquatic environments. *Organic Geochemistry* **28,** 111-118.

Harris, R.P. (1994) Zooplankton grazing on the coccolithophore *Emiliania huxleyi* and its role in inorganic carbon flux. *Marine Biology* **119**, 431-439.

Harris, P.G., Carter, J.F., Head, R.N., Harris, R.P., Eglinton ,G. and Maxwell, J.R. (1995a) Identification of chlorophyll transformation products in zooplankton faecal pellets and marine sediment extracts by liquid chromatography/mass spectrometry atmospheric pressure chemical ionisation. *Rapid Communications in Mass Spectrometry* **9**, 177-1183.

Harris, P.G., Pearce, G.E.S., Peakman, T.M. and Maxwell, J.R. (1995b). A widespread and abundant chlorophyll transformation product in aquatic environments. *Organic Geochemistry* **23**, 183-187.

Harvey, H.R., Eglinton, G., O'Hara, S.C.M. and Corner, D.S. (1987) Biotransformation and assimilation of dietary lipids by *Calanus* feeding on a dinoflagellate. *Geochimica et Cosmochimica Acta* **51**, 3031-3040.

Harvey, H.R., O'Hara, S.C.M., Eglinton, G. and Corner, D.S. (1989) The comparative fate of dinosterol and cholesterol in copepod feeding: Implications for a conservative molecular biomarker in the marine water column. *Organic Geochemistry* **14**, 635-641.

Harvey, H.R. and McManus, G.B. (1991) Marine ciliates as a widespread source of tetrahymanol and hopan-3β-ol in sediments. *Geochimica et Cosmochimica Acta* **55**, 3387-3390.

Hawkins, A.J.S., Bayne, B.L., Mantoura, R.F.C. and Llewellyn, C.A. (1986) Chlorophyll degradation and absorption through the digestive system of the blue mussel *Mytilus edulis*. *Journal of Experimental Marine Biology and Ecology* **96**, 213-223.

Head, E.J.H. (1992) Gut pigment accumulation and destruction by arctic copepods *in vitro* and *in situ*. *Marine Biology* **112**, 583-592.

Head, E.J.H. and Harris, L.R. (1992) Chlorophyll and carotenoid transformation and destruction by *Calanus* spp. grazing on diatoms. *Marine Ecology Progress Series* **86**, 229-238.

Head, E.J.H. and Harris, L.R. (1994) Feeding selectivity by copepods grazing on natural mixtures of phytoplankton determined by HPLC analysis of pigments. *Marine Ecology Progress Series* **110**, 75-83.

Head, E.J.H. and Harris, L.R. (1996) Chlorophyll destruction by *Calanus* spp. grazing on phytoplankton: kinetics, effects of ingestion rate and feeding history and a mechanistic interpretation. *Marine Ecology Progress Series* **135**, 223-235.

Heinbokel, J.F. and Beers, J.R. (1979) Studies on the functional role of tintinnids of the southern California Bight. III Grazing impact of natural assemblages. *Marine Biology* **52**, 23-32.

Hendry, G.A.F, Haughton, J.D. and Brown, S.B. (1987) The degradation of chlorophyll - a biological enigma. *New Phytologist* **107**, 255-302.

Hofmann, E.E., Klinck, J.M. and Paffenhofer, G-A. (1981) Concentration and vertical flux of zooplankton fecal pellets on a continental shelf. *Marine Biology* **61**, 327-335.

Hynninen P.H. (1991) Chemistry of chlorophylls: modifications. In: *The Chlorophylls*, ed. H. Scheer, pp.145. CRC Press, Boca Ranton, USA. p145

Jeffrey, S.W. (1976) A report of green algal pigments in the central North Pacific Ocean. *Marine Biology* **37**, 33-37.

Jeffrey, S.W. and Hallegraeff, G.M. (1980a) Studies of phytoplankton species and photosynthetic pigments in a warm-core eddy of the East Australian Current I. Summer populations. *Marine Ecology Progress Series* **3**, 285-294.

Jeffrey, S.W. and Hallegraeff, G.M. (1980b) Photosynthetic pigment profiles and phytoplankton species in a warm-core eddy of the Eastern Australian Current. II. A note on methodology. *Marine Ecology Progress Series* **3**, 295-301.

Jeffrey, S.W. and Hallegraeff, G.M. (1987) Chlorophyllase distribution in ten classes of phytoplankton: a problem for chlorophyll analysis. *Marine Ecology Progress Series* **35**, 293-304.

Jeffrey, S.W. and Wright, S.W. (1994) Photosynthetic pigments in the Prymnesiophyceae. In: *The Haptophyte Algae*, eds. J.C. Green and B.S.C. Leadbeater, pp. 111-132. Clarendon Press, Oxford.

Jeffrey, S.W. and Mantoura, R.F.C. (1997) Chapter 1: Development of pigment methods for oceanography: SCOR-supported Working Groups and objectives. In *Phytoplankton Pigments In Oceanography*, eds. S.W. Jeffrey, R.F.C. Mantoura and S.W. Wright, pp19-36. UNESCO Publishing, France.

Jeffrey, S.W., Mantoura, R.F.C. and Bjørnland, T. (1997) Part IV. Data for the identification of 47 key phytoplankton pigments. In *Phytoplankton Pigments In Oceanography*, eds. S.W. Jeffrey, R.F.C. Mantoura and S.W. Wright, pp449-559. UNESCO Publishing, France.

Jeffrey, S.W. and Vesk, M. (1997) Chapter 2: Introduction to marine phytoplankton and their pigment signatures. In *Phytoplankton Pigments In Oceanography*, eds. S.W. Jeffrey, R.F.C. Mantoura and S.W. Wright, pp37-84. UNESCO Publishing, France.

Johnson., P.W., Xu, H. and Sieburth, J.McN. (1982) The utilization of chroococcoid cyanobacteria by protozooplankters but not by calanoid copepods. *Ann. Inst. océanogr., Paris.* **58**, 297-308.

Karuso, P., Berquist, P.R., Buckleton, J.S., Cambie, R.C., Clark, G.R. and Rickard, C.E.F. (1986) 13^2,17^3-cyclopheophorbide enol, the first porphyrin isolated from a sponge. *Tetrahedron Letters* **27**, 2177-2178.

Keely, B.J. (1989) Early diagenesis of chlorophyll and chlorin pigments. Ph D.Thesis. University of Bristol.

Keely, B.J., Brereton, R.G. and Maxwell, J.R. (1987) Occurrence and significance of pyrochlorins in a lake sediment. *Organic Geochemistry* **13**, 801-805.

Keely, B.J., Prowse, W.G. and Maxwell, J.R. (1990) The Treibs Hypothesis: An evaluation based on structural studies. *Energy and Fuels* **4**, 628-634.

Keely, B.J. and Maxwell, J.R. (1991) Structural characterisation of the major chlorins in a recent sediment. *Organic Geochemistry* **17**, 663-669.

Keely, B.J., Harris, P.G., Popp, B.N. Hayes, J.M., Meischner, D. and Maxwell, J.R. (1994) Porphyrin and chlorin distributions in a Late Pliocene lacustrine sediment. *Geochimica et Cosmochimica Acta* **58**, 3691-3701.

King, L.L. (1995) A mass balance of chlorophyll degradation product accumulation in Black Sea sediments. *Deep-Sea Research Part I - Oceanographic research papers* **42**, 919-942.

King, L.L. and Repeta, D.J. (1991) Novel pyrophaeophorbide steryl esters in Black Sea sediments. *Geochimica et Cosmochimica Acta* **55**, 2067-2074.

King, L.L. and Repeta, D.J. (1994) Phorbin steryl esters in Black Sea sediment traps and sediments: a preliminary evaluation of their palaeooceanographic potential. *Geochimica et Cosmochimica Acta* **58**, 4389-4399.

King, L.L. and Wakeham, S.G. (1996) Phorbin steryl ester formation by macrozooplankton in the Sargasso Sea. *Organic Geochemistry* **24**, 581-585.

Kiørboe, T., Møhlenberg, F. and Nicolajsen, H. (1982) Ingestion rate and gut clearance in the planktonic copepod *Centrophages hamatus* (Lilljeborg) in relation to food concentration and temperature. *Ophelia* **21**, 181-194.

Kiørboe,T. and Tiselius, P.T. (1987) Gut clearance and pigment destruction in herbivorous copepod, *Acartia tonsa*, and the determination of in situ grazing rates. *Journal of Plankton Research* **9**, 525-534.

Klein, B., Gieskes, W.W.C., and Kraay, G.G. (1986) Digestion of chlorophylls and carotenoids by the marine protozoan *Oxyrrhis marina* studied by HPLC analysis of algal pigments. *Journal of Plankton Research* **8**, 827-836.

Kowalewska, G. (1994) Steryl chlorin esters in sediments of the southern Baltic Sea. *Netherlands Journal of Aquatic Ecology* **28**, 149-156.

Kowalewska G., Winterhalter B. Talbot H.M., Maxwell J.R. and Konat J. (1999) Chlorins in sediments of the Gotland Deep (Baltic Sea). *Oceanologia* **41**, 81-97.

Kozyrev, A.N., Dougherty, T.J. and Pandey, R.K. (1998) LiOH promoted allomerization of pyropheophorbide *a*. A convenient synthesis of 13^2-oxopyropheophorbide *a* and its unusual enolisation (1998) *Chemical Communications,* 481-482.

Landry, M.R. and Hassett, R.P. (1982) estimating the grazing impact of marine microzooplankton. *Marine Biology* **67**, 283-288.

Laureillard, J., Pinturier, L., Fillaux, J. and Saliot, A. (1997) Organic geochemistry of marine sediments of the Subantarctic Indian Ocean sector: Lipid classes - sources and fate. *Deep-Sea Research Part II - Topical Studies In Oceanography* **44**, 1085-1108.

Lin, D.S., Ilias, A.M., Conner, W.E., Caldwell, R.S., Corey, H.T. and Davies, G.D. (1982) Composition and biosynthesis of sterols in selected marine phytoplankton. *Lipids* **17**, 818-824.

Lopez, M.D.G., Huntley, M.E. and Sykes, P.F. (1988) Pigment destruction by *Calanus pacificus*: impact on the estimation of water column fluxes. *Journal of Plankton Research* **10**, 715-734.

Lorenzen, C.J., Shuman, F.R. and Bennett, J.T. (1981) In situ calibration of a sediment trap. *Limnology and Oceanography* **26**, 580-585.

Louda, J.W., Li, J., Liu, L. Winfree, M.N. and Baker, E.W. (1998) Chlorophyll-*a* degradation during cellular senescence and death. *Organic Geochemistry* **29**, 1233-1251.

Ma, L. and Dolphin, D. (1995) Asymmetric hydroxylation of chlorophyll derivatives: A facile entry to both diastereomers of chlorophyllone *a*. *Tetrahedron Asymmetry* **6**, 313-316.

Mantoura, R.F.C. and Llewellyn, C.A. (1983) The rapid determination of algal chlorophyll and carotenoids pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. *Analytica Chimica Acta* **151**, 297-314.

Mattox, K.R. and Stewart, K.D. (1984) Classification of green algae: a concept based on comparative cytology. In: *Systematics of the Green Algae*, eds. D.E.G. Irvine and D.M. John. Academic Press, London, pp 29-72.

Naylor, C.C. and Keely, B.J. (1998) Sedimentary purpurins: oxidative transformation products of chlorophylls. *Organic Geochemistry* **28**, 417-422.

Nelson, J.R. (1989) Phytoplankton pigments in macrozooplankton faeces: variability in carotenoid alterations. *Marine Ecology Progress Series* **52**, 129-140.

Nelson, J.R. (1993) Rates and possible mechanism of light-dependent degradation of pigments in detritus derived from phytoplankton. *Journal of Marine Research* **51**, 155-179.

Nöthig, E-M. and von Bodungen, B. (1989) Occurrence and vertical flux of faecal pellets of probably protozoan origin in the southeastern Weddell Sea (Antarctica). *Marine Ecology Progress Series* **56**, 281-289.

Ocampo, R. and Repeta, D.J. (1999) Isolation and structural determination of purpurin-18 (as methyl ester) from sedimentary organic matter. *Organic Geochemistry* **30**, 189-193.

Ocampo, R., Sachs, J.P. and Repeta, D.J. (1999) Isolation and structure determination of the very unstable 13^2, 17^3-cylopheophorbide *a* enol from recent sediments. *Geochimica et Cosmochimica Acta*. In press.

Otsuki, A., Kaneda, Y. and Hashimoto S. (1993) Identification and significance of pyrochlorins in fecal pellets of the marine malacostrian crustaceans *Heptacarpus rectirostris* and *Palaemon pacificus*. *Marine Biology* **115**, 463-467.

Ourisson, G., Albrecht, P. and Rohmer, M. (1979) The Hopanoids: Palaeochemistry and biochemistry of a group of natural products. *Pure and Applied Chemistry* **51**, 709-729.

Pasternak, A.F. and Drits, A.V. (1988) Possible degradation of chlorophyll-derived pigments during gut passage of herbivorous copepods. *Marine Ecology Progress Series* **49**,187-190.

Pearce, G.E.S. (1994) Occurrence, characterisation and significance of sedimentary steryl chlorin esters. Ph.D. Thesis. University of Bristol.

Pearce, G.E.S., Keely, B.J., Harradine, P.J., Eckardt, C.B. and Maxwell, J.R. (1993) Characterisation of naturally occurring steryl esters derived from chlorophyll *a*. *Tetrahedron Letters* **34**, 2989-2992.

Pearce, G.E.S., Harradine, P.J., Talbot, H.M. and Maxwell, J.R. (1998) Sedimentary sterols and steryl chlorin esters: distribution differences and significance. *Organic Geochemistry* **28**, 3-10.

Penry, D.L. and Frost, B.W. (1991) Chlorophyll-*a* degradation by *Calanus pacificus*: Dependence on ingestion rate and digestive acclimation to food resources. *Limnology and Oceanography* **36**, 147-159.

Pilskaln, C.H and Honjo, S. (1987) The faecal pellet fraction of biogeochemical particle fluxes to the deep sea. *Global Biogeochemical Cycles* **1**, 31-48.

Pond, D.W., Harris, R.P. and Brownlee, C. (1995) A microinjection technique using a pH-sensitive dye to determine the gut pH of *Calanus helgolandicus*. *Marine Biology* **123**, 75-79.

Porra, R.J., Pfündel, E.E. and Engel, N. (1997) Metabolism and functions of photosynthetic pigments. In *Phytoplankton Pigments In Oceanography* (Edited by Jeffrey S.W., Mantoura R.F.C. and Wright S.W.) pp84-126. UNESCO Publishing, France.

Prahl, F.G., Eglinton, G., Corner, E.D.S., O'Hara, S.C.M. and Forsberg, T.E.V. (1984) Changes in plant lipids during passage through the gut of *Calanus*. *Journal of the Marine Biological Association U.K.* ***I*64**, 317-334.

Prowse, W.G., Chicarelli, M.I., Keely, B.J., Kaur, S. and Maxwell, J.R. (1987) Characterisation of fossil porphyrins of the "di-DPEP" type. *Geochimica et Cosmochimica Acta* **51**, 2875-2877.

Prowse, W.G. and Maxwell, J.R. (1991) High molecular weight chlorins in a lacustrine shale. *Organic Geochemistry* **17**, 877-886.

Quirke, J.M.E. and Maxwell, J.R. (1980) Petroporphyrins-III. Characterisation of a C_{32} aetioporphyrin from Gilsonite as the bis(porphyrinato-mercury(II)acetato) mercury(II) complex. Origin and significance. *Tetrahedron* **36**, 3453-3456.

Repeta, D.J. (1995) Geochemistry of pyrophaeophorbide steryl esters. in *Organic Geochemistry: Developments and Applications to Energy, Climate, Environment, and Human History*, eds. J.O. Grimalt and C. Dorronsoro, pp. 1142-1143. AIGOA, San Sebastian, Spain.

Repeta, D.J. and Gagosian, R.B. (1987) Carotenoid diagenesis in recent sediments - I. the Peru continental Shelf (15°S, 75°W). *Geochimica et Cosmochimica Acta* **51**, 1001-1009.

Riffé-Chalard, C., Verzegnassi, L. and Gülaçar, F.O. (1999). Pheophorbide *a* steryl esters in an oxic surface sediment. Conference Abstract. In press .

Rontani, J.F., Baillet, G. and Aubert, C. (1991) Production of acyclic isoprenoid compounds during the photodegradation of chlorophyll *a* in sea water. *Journal of Photochemistry and Photobiology* **59**, 369-377.

Roy, S., Harris, R.P. and Poulet, S.A. (1989) Inefficient feeding by *Calanus helgolandicus* and *Temora longicornis* on *Cosciondiscus wailesii*: quantitative estimation using chlorophyll-type pigments and effects on dissolved free amino acids. *Marine Ecology Progress Series* **52**, 145-153.

Sakata, K., Yamamoto, K., Ishikawa, H., Yagi, A., Etoh, H. and Ina, K. (1990) Chlorophyllone *a*, a new phaeophorbide -a related compound isolated from *Ruditapes philippinarum* as an antioxidative compound. *Tetrahedron Letters* **31**, 1165-1168.

Sakata K., Yamamoto, K. and Watanabe, N. (1994) Chapter 18. Antioxidative compounds from marine organisms. *American Chemical Society Symposium Series* **547**, 164-182.

Scheer, H. (1991). Structure and occurrence of chlorophylls. In: *The Chlorophylls* ed. H. Scheer, pp. 3-30. CRC Press, Boca Raton.

Schoch, S., Scheer, H., Schiff, J.A., Rüdiger, W. and Siegelman, H.W. (1981) Pyrophaeophytin *a* accompanies phaeophytin *a* in darkened light grown cells of *Euglena*. *Zertschrift fur Naturforschung C - A journal of Biosciences* **36**, 827-833.

Shioi, Y., Tatsumi, Y. and Shimokawa, K. (1991) Enzymatic degradation of chlorophyll in *Chenopodium album*. *Plant Cell Physiology* **32**, 87-93.

Shuman, F.R. and Lorenzen ,C.J. (1975) Quantitative degradation of chlorophyll by a marine herbivore. *Limnology and Oceanography* **20**, 580-586.

Silver, M.W., and Alldredge, A.L., (1981) Bathypelagic marine snow: deep-sea algal and detrital community. *Journal of Marine Research* **39**, 501-530.

Sinninghe-Damsté J.S., Kenig, F., Koopmans, M.P., Köster, J., Schouten, S., Hayes, J.M. and de Leeuw J.W. (1995) Evidence for gammacerane as an indicator of water column stratification. *Geochimica et Cosmochimica Acta* **59**, 1895-1900.

SooHoo, J.B. and Kiefer, D.A. (1982) Vertical distribution of phaeopigments. I. A simple grazing and photooxidative scheme for small particles *Deep-Sea Research Part A - Oceanographic Research Papers* **29**, 1553-1551.

Spooner, N., Keely, B.J. and Maxwell, J.R. (1994a) Biologically mediated defunctionalization of chlorophyll in the aquatic environment - I. Senescence/decay of the diatom *Phaeodactylum tricarnutum*. *Organic Geochemistry* **21**, 509-516.

Summons, R.E., Thomas, J., Maxwell, J.R. and Boreham, C.J. (1992) Secular and environmental constraints on the occurrence of dinosterane in sediments. *Geochimica et Cosmochimica Acta* **56**, 2437-2444.

Spooner, N., Harvey, H.R., Pearce, G.E.S., Eckardt, C.B. and Maxwell, J.R. (1994b) Biological defunctionalization of chlorophyll in the aquatic environment - II. action of endogenous algal enzymes and aerobic bacteria. *Organic Geochemistry* **22**, 773-780.

Stauber, J.L. and Jeffrey, S.W. (1988) Photosynthetic pigments in fifty one species of marine diatoms. *Journal of Phycology* **24**, 158-172.

Stevens, C.J. and Head, E.J.H. (1998) A model of chlorophyll *a* destruction by *Calanus* spp. And implications for the estimation of ingestion rates using the gut fluorescence method. *Marine Ecology Progress Series* **171**, 187-198.

Strom, S.L. (1991) Growth and grazing rates of the herbivorous dinoflagellate *Gymnodinium* sp. from the open subarctic Pacific Ocean. *Marine Ecology Progress Series* **78**, 103-113.

Strom, S.L. (1993) Production of pheopigments by marine protozoa: results of laboratory experiments analysed by HPLC. *Deep-Sea Research I.-Oceanographic Research papers* **40**. 57-80.

Svec, W.A. (1991) The distribution and extraction of the chlorophylls. In: *The Chlorophylls*. ed. H. Scheer H., pp. 89-102. CRC Press, Boca Ranton, USA.

Svoboda, J.A. and Feldlaufer, M.F. (1991) Neutral sterol metabolism in insects. *Lipids* **26**. 614-618.

Teece, M. (1994) Biodegradation of algal lipids and significance for sediment studies. Ph.D. Thesis, University of Bristol, UK.

ten Haven, H.L., Rohmer, M., Rullkötter, J. and Bisseret, P. (1989) Tetrahymanol, the most likely precursor of gammacerane, occurs ubiquitously in marine sediments. *Geochimica et Cosmochimica Acta* **53**, 3073-3079.

Treibs, A. (1936) Chlorophyll und häminderivate in organischen mineralstoffen. *Angew. Chem.* **49,** 682-686.

van den Hoek, C., Mann, D.G. and Jahns, H.M. (1995) 14. Haptophyta (=*Prymnesiophyta)*. In: *ALGAE. An Introduction to Phycology*. Cambridge University Press.

Venkatesan, M.I. (1989) Tetrahymanol - its widespread occurrence and geochemical significance. *Geochimica et Cosmochimica Acta* **53**, 3095-3101.

Vernet, M. and Lorenzen, C.J. (1987a) The relative abundance of phaeophorbide *a* and phaeophytin *a* in temperate marine waters. *Limnology and Oceanography* **32**, 352-358.

Vernet, M. and Lorenzen, C.J. (1987b) The presence of chlorophyll *b* and the estimation of phaeopigments in marine phytoplankton. *Journal of Plankton Research* **9**, 255-265.

Villanueva, J., Grimalt, J.O., De Wit R., Keely, B.J. and Maxwell, J.R. (1994) Sources and transformations of Chlorophylls and carotenoids in a monomictic sulphate-rich karstic lake environment. *Organic Geochemistry* **22**, 739-757.

Volkman, J.K. (1986) A review of sterol markers for marine and terrigenous organic matter. *Organic Geochemistry* **9**, 83-99.

Volkman, J.K., Barrett, S.M., Dunstan, G.A. and Jeffrey, S.W. (1993) Geochemical significance of the occurrence of dinosterol and other 4-methyl sterols in a marine diatom. *Organic Geochemistry* **20**, 7-15.

Volkman, J.K., Barrett, S.M., Dunstan, G.A. and Jeffrey S.W. (1994) Sterol biomarkers for microalgae from the green algal class Prasinophyceae. *Organic Geochemistry* **21**, 1211-1218.

Voss, M. (1991) Contents of copepod faecal pellets in relation to food supply in Kiel Bight and its effect on sedimentation rate. *Marine Ecology Progress Series* **75**, 217-225.

Wakeham, S.G. (1987) Steroid geochemistry in the oxygen minimum zone of the eastern tropical North Pacific Ocean. *Geochimica et Cosmochimica Acta* **51**, 3051-3069.

Wakeham, S.G. (1989) Reduction of sterols to stanols in particulate matter at oxic-anoxic boundaries in seawater. *Nature* **342**, 787-790.

Watanabe, N., Yamamoto, K., Ihshikawa, H., Akihito, Y., Sakata, K., Brinen, L.S. and Clardy, J. (1993) New chlorophyll-a-related compounds isolated as antioxidants from marine bivalves. *Journal of Natural Products* **56**, 305-317.

Welschmeyer, N.A. and Lorenzen, C.J. (1985) Chlorophyll budgets: zooplankton grazing and phytoplankton growth in a temperate fjord and the Central Pacific gyres. *Limnology and Oceanography* **30**, 1-21.

Wolff, G.A., Murray, M., Maxwell, J.R., Hunter, B.K. and Sanders, J.K.M. (1983) 15,17-Butano-3,8-diethyl-2,7,12,18-tetraramethylporphyrin - a novel naturally occurring tetrapyrrole. *Journal of the Chemical Society, Chemical Communications*, 922-924.

Wolff, G.A., Lamb, N.A. and Maxwell, J.R. (1986) The origin and fate of 4-methyl steroids II. Dehydration of stanols and occurrence of C_{30} 4-methyl steranes. *Organic Geochemistry* **10**, 965-974.

Woolley, P.S., Keely, B.J. and Hester, R.E. (1997) Surface-enhanced resonance Raman spectroscopic identification of chlorophyll *a* allomers. *Journal of the Chemical Society. Perkin Transactions 2*, 1731-1734.

Woolley, P.S., Moir, A., Hester, R.E. and Keely, B.J. (1998) A comparative study of the allomerization reaction of chlorophyll *a* and bacteriochlorophyll *a*. *Journal of the Chemical Society. Perkin Transactions 2,* 1833-1839.

Wright, S.W., Jeffrey, S.W. Mantoura, R.F.C., Llewellyn, C.A., Bjørnland, T., Repeta, D. and Welschmeyer, N.A. (1991). Improved method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Marine Ecology Progress Series* **77**, 183-196.

Ziegler, R., Blaheta, A., Guha, N. and Schonegge, B. (1988) Enzymatic formation of phaeophorbide and pyrophaeophorbide during chlorophyll degradation in a mutant of *Chlorella fusca* SHIHIRA et KRAUS. *Journal of Plant Physiology* **132**, 327-332.